AUTOIMMUNE RESPONSES AGAINST CULTIVATED THYROCYTES

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For my son and husband

STATEMENT OF ORIGINALITY

The work described in this thesis is original and was carried out by myself under the supervision of Dr. P. J. McCullagh in the Division of Clinical Sciences. Unless otherwise stated, it is the original work of the author and has neither been presented nor is it currently being presented for any other degree.

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ABSTRACT

This thesis reports the results of *in vitro* investigations of the mechanisms of negative regulation and pathogenesis of autoimmune responses against cultivated thyrocytes. The first of three aspects to be examined was the extent to which deprivation of exposure to thyroid-specific antigens during development would be reflected in specific autoreactivity by lymphocytes isolated from the affected animals. The second issue related to identification of mechanisms of negative regulation of autoreactivity that could explain resistance to induction of abnormal autoimmune responses in normal individuals. The remaining experiments examined possible pathogenic mechanisms of autoantigen recognition during the induction of abnormal autoimmune responses.

Experimental interference, at an earlier stage of development, with the acquisition of self-tolerance to thyroid antigens in foetal Merino lambs and DA rats has been shown to lead to the appearance of autoreactivity on the part of their lymphocytes against cultivated autologous or syngeneic thyrocytes respectively. The presence of autoreactive lymphocytes has been demonstrated by observation of their cytotoxic responses against cultivated thyrocyte monolayers.

Normal lymphocytes from untreated foetal lambs regularly down regulated the anti-thyrocyte autoreactivity expressed by lymphocytes from their thyroidectomized identical co-twins. Similarly, lymphocytes from normal DA rats exerted an effective negative regulation on the autoimmune cytotoxicity, for cultivated thyrocytes, of lymphocytes from rats exposed to ^{131}I during foetal life. These results suggested that a capacity for negative regulation of autoimmunity on the part of normal lymphocyte populations could be of practical importance in the lack of autoreactivity in normal individuals. In the case of anti-thyroid autoimmunity, both cytotoxic and regulatory cells were from the $CD8^+$ lineage.

Implantation of thyroid allografts in thyroidectomized foetal lambs failed to prevent the development of autoimmune responses against cultivated thyrocytes demonstrable later in foetal life. However, allografting induced tolerance of recipient lymphocytes towards cultivated thyrocytes from the allograft donor. These results

suggest that a complex of peripheral self-antigen and the MHC, were recognised by developing immunocomponent cells. It appears likely that MHC antigens played an important role both in the induction of autoimmune responses against peripheral antigens and in their negative regulation. Whilst the expression of MHC class II antigens by thyrocytes subject to autoimmune attack was demonstrated in these experiments, it was unlikely to be a primary cause of initiation of autoimmune responses. The alterative explanation was that expression of MHC class II antigens on thyrocytes was an *effect* of exposure to autoreactive lymphocytes.

ABBREVIATIONS

ABC	avidin-biotin complex
ACTH	adrenocorticotropic hormone
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CBB-R250	coomassie brilliant blue R-250
BSA	bovine serum albumin
DAB	diaminobenzidine tetrahydrochlorede
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EAT	experimental autoimmune thyroiditis
FACS	fluorescence activated cell sorting
FCA	Freund's complete adjuvant
F15	modified Eagle's basal medium F15
FITC	fluoresceinisothiocyanate
GD	Graves' disease
HBSS	Hanks balanced salt solution
HD	Hashimoto's disease
H & E	haematoxylin and eosin
HLA	human leucocyte antigen
IFN-γ	interferon-gamma
MHC	major histocompatibility complex
MSH	melanophore stimulating hormone
NSB	non specific background
NZB	New Zealand Black
NZW	New Zealand White
OVA	ovalbumin
PBS	phosphate buffered saline
PVDF	polyvinylidene difluoride

RIBC	radioiodide bound to cells
RT	room temperature
SDE	subacute demyelinating encephalomyelitis
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
TCA	trichloroacetic acid
TCR	T cell receptor
TEC	thyroid epithelial cells
TEMED	tetramethylethylenediamine
Tg	thyroglobulin
TSH	thyroid stimulating hormone

CONTENTS

CHAPTER 1: INTRODUCTION

1.1. Content of the thesis

This thesis will present the results of a series of experiments using a recently developed experimental autoimmune animal model that permits analysis of *in vitro* autoimmune responses by lymphocytes against cultivated thyrocytes. These experiments have as their objective the investigation of the mechanisms whereby autoimmunity against cultivated thyrocytes is induced in the course of immunological maturation, in the absence of thyroid determinants, but is negatively regulated in normal animals.

To explain the rationale underlying the studies reported in this thesis, I will review the historical development of current theories about self-tolerance, autoimmunity and the relationship between these phenomena. After reviewing some earlier experimental models of autoimmune responses, the new experimental models used in this thesis will be discussed. Particular attention will be given to investigation of the regulation of autoimmunity which will be a central issue in this thesis. Finally, as thyrocytes are the target antigens of the autoimmune responses in the present studies, some specific details of earlier investigations of autoimmune thyroiditis will be outlined.

1.2. Theories of self-tolerance

As pathological autoimmune responses are usually considered to represent the failure of mechanisms that normally modulate abnormal immune responses against self-antigens, it is appropriate to consider, in the first instance, the nature of these normal homeostatic (or self-tolerant) mechanisms.

In 1900, Ehrlich and Morgenroth (cited by Naparstek and Schwartz, 1988) proposed that there was a requirement that the immune system would not normally react to self. Their proposition was based on two observations. The first of these was that, when inoculated with blood cells of a foreign species, an animal always produced a specific haemolysin. The second was that production of haemolysin did not occur in an

individual which had been given autologous blood. In 1945, Owen advanced the suggestion that naturally occurring tolerance was not only genetically encoded, but could also be an epigenetic self-recognition process. Owen reported that the majority of dizygotic cattle twins which had anastomoses between their placental blood vessels retained a proportion of red cells which were genetically self, mixed with red cells derived from the co-twin. Each animal appeared to be unresponsive to red cells from its co-twin. Since these reports appeared, many theories have been proposed to explain self-tolerance. Three major theories remain current. These are clonal deletion, clonal anergy and immunosuppression.

Burnet (1957) introduced the concept of clonal immune responses to explain the induction of self-tolerance. His hypothesis was that both auto-antigens and the corresponding reactive immunologically component cells were genetically coded for in normal individuals. Autoantigens could stimulate cells of the appropriately reactive clones to proliferation and antibody production. During early embryonic development, a very large number of clones of immunocytes were generated. It was proposed that contact of immunologically competent cells with the corresponding antigen at a sufficiently early stage of development would result in the elimination of those cells. Burnet concluded that immune tolerance to self antigens was achieved by deletion of any clonal foetal immunocytes that encountered their specific antigens.

More than 10 years later, some doubts about the universal applicability of the "clonal deletion" theory arose following study of the cellular characteristics of the development of self-tolerance. Chiller *et al.* (1971) found that unresponsiveness of normal mice to human gamma globulin could be established much more readily in thymus-derived cells than in bone marrow-derived cells. The former were found to have a lower threshold dose for tolerance induction than the latter. Tolerance was much more rapidly lost in the B cell compartment. Chiller *et al.* surmised that thymus cells might be rendered unresponsive simply by passive interaction of tolerogen with cells. In contrast, bone marrow cells, it was suggested, might require an active process, such as cell division, differentiation, or antibody formation, to reach a tolerogen-sensitive stage. In addition, the induction of unresponsiveness in the bone marrow population might

require a higher level of tolerogen. Hence, low concentrations of antigen might only induce thymus-derived cell tolerance, but not tolerance in bone marrow-derived cells.

On the basis of these and other results, Nossal (1975) proposed a "clonal abortion" concept for B cell tolerance. It was pointed out that the models associated with unresponsiveness of B cells usually involved either the administration of relatively high doses of soluble, aggregate-free and poorly immunogenic antigens, or the use of highly polymeric antigens with multiple antigenic determinants in supra-immunogenic concentrations. In some of these models, B cells could have been blockaded by the antigens effectively promoting their unresponsiveness. The concept of "clonal abortion" implied that immunocomponent cells, at a particular stage of their differentiation, could be permanently switched off or eliminated by blockade of antigens on the receptor if antigen was encountered in appropriate concentration. In 1980, Nossal (1980) redefined and expanded his "clonal abortion" theory. He postulated that at some stage in their differentiation from stem cell to mature, antibody-forming precursor cells, B lymphocytes pass through a phase during which contact with even low doses of antigen induces tolerance rather than immunity. In other words, he suggested that immature B cells received some negative signal as a result of which they were inactivated without being eliminated. Such a hypothesis was supported by the ease of inducing tolerance in neonatal spleen cells and bone marrow cells. Significantly, spleens of adult animals were found to contain a minor subset of immature B cells that could be tolerated as readily as neonatal B cells (Nossal, 1980). This finding suggested that susceptibility to tolerance induction was not a property unique to cells in neonatal animals but was a feature of immature B cells. In general, such cells were continuously produced throughout life. Hence, he proposed "clonal anergy" as a more accurate description of tolerance induction (Nossal, 1980).

Another advance, during the 1970s, was the demonstration (McCullagh, 1970a, b; Gershon and Kondo, 1971) that some models of tolerance involved the development of T lymphocytes capable of suppressing immune responses. McCullagh reported that cells with specific inhibitory capacity were present in some rats manifesting artificially induced tolerance of non-self determinants. Gershon and Kondo showed that spleen

cells transferred from tolerant to non-tolerant individuals were able specifically to inhibit humoral immune responses of the non-tolerant individual in vivo. The cells mediating this effect were shown to be thymus-derived and antigen-specific. They suggested that the thymus might release a humoral factor that influenced the induction of unresponsiveness in bone marrow cells, or that thymus-bone marrow cell interaction might have to occur for induction of tolerance. Although the exact nature of these phenomena remains controversial, all three contributions highlight the potential importance of suppression in the development of self-tolerance.

Thereafter, the phenomenon of antigen-specific T cell mediated regulation has been repeatedly reported (reviewed by Dorf *et al.* 1984). A number of mechanisms have been suggested to explain T cell mediated modulation. Examples of such mechanisms have included release of soluble antigen-specific T suppressor factors (Green *et al.* 1991), cell-mediated cytotoxicity (Sun *et al.* 1988), cytokine-mediated suppression (Panels *et al.* 1992), sending of a non-cytolytic negative signal from a T suppressor cell to its target cell after cell-cell contact (Fink *et al.* 1988) and anti-idiotypic networks (Stab *et al.* 1990). Among these explanations, proposals for the existence of an "idiotypic network" have received much attention. This possibility was first raised by Jerne (1974). He hypothesised that, if lymphocytes could recognise a whole range of foreign antigenic determinants, they should be able to recognise the idiotype on other lymphocytes. They would therefore form a large network or series of networks depending upon idiotype-anti-idiotype recognition between lymphocytes of the various T- and B-subsets. This theory has been supported by several experimental results. For example, Kearney and Vakil (1986) showed that natural idiotypic interactions between neonatal B cells played an important role in the early establishment of the B cell repertoire which was subsequently expressed in adult mice. Stab *et al.* (1990) have isolated $CD4^+$ idiotype-specific (non-antigen specific) T cell lines and clones. These T cells inhibited *in vitro* proliferation and antibody secretion by B cells. This strongly suggested the existence of an important role for anti-idiotypic reactivity in the modulation of immune responses.

In contrast to "clonal deletion" types of theory, Coutinho (1989) suggested that, self-tolerance, as explained by suppression and idiotypic network theories, might represent the coexistence at physiological equilibrium of immune-activated components with somatic self molecules. The immune-activated components of such systems would include self-reactive cells and specific suppressor cells carrying anti-idiotypic determinants. Self-tolerance has been produced by means of stimulation of the development of suppressor cell and idiotypic network mediated physiological autoimmune responses (Coutinho, 1989).

Self-tolerance could, theoretically at least, be achieved by means of either central (intrathymic) or peripheral (extrathymic) mechanisms. Thymic tolerance has been considered to be operative in the case of those antigens that could be secreted into the bloodstream, or expressed on lymphoid cells, and transported into the thymus to induce tolerance to those antigens (Lo, 1992). Both "clonal deletion" and "clonal anergy" theories have been suggested as responsible for the induction of intrathymic tolerance. Marrack *et al.* (1988) showed that thymic epithelium was capable of inducing deletion of T cells. However, the deletion induced by the epithelium was not as complete as the deletion induced by bone marrow derived cells. This could be attributable either to the lack of a strong antigen-presenting function on the part of the epithelium because of low levels of expression of MHC molecules, or to failure of the epithelium to express sufficient levels of antigen with affinity with the specific Vβ-chain of T cells (Marrack *et al.* 1988). On the contrary, Ramsdell *et al.* (1989) proposed that non-deletion mechanisms could be involved in intra-thymic self-tolerance to antigens expressed on the thymic stromal elements. T cells appeared to be tolerant to host antigens, but with the occurrence of little clonal deletion. Hence, they suggested that the thymus was capable of inducing tolerance by at least 2 distinct mechanisms, namely "clonal deletion" and "clonal anergy".

However, many antigens, for example pancreatic islet cell antigens, are characterised by being expressed only on non-lymphoid peripheral tissues and by not being secreted in large amounts into the bloodstream (Lo, 1992). Posselt *et al.* (1992) injected pancreatic islet cells directly into the thymus of BioBreeding (BB) rats, a strain

which spontaneously develops autoimmune insulitis mediated by islet-specific T cells. However, after this injection, autoimmune diabetes was prevented. A more likely explanation for their finding may be that intrathymic transplantation of islets alters T cell development by promoting the deletion or functional inactivation of antigen-specific clones before their migration to the periphery. This would contrast with the normal situation in which tissue-specific autoantigens on pancreatic islet cells are not transported to the thymus. Tolerance to such "peripheral" antigens could involve non-deletion mechanisms.

"Clonal deletion" types of theory have still been used to explain the mechanisms of peripheral tolerance. Webb *et al.* (1990) examined tolerance of mature peripheral T cells to minor lymphocyte stimulation molecules. Exposure of mature T cells to minor lymphocyte stimulating antigens *in vivo* led to specific tolerance and disappearance of minor lymphocyte stimulating antigen-reactive T cells. The host rats were subjected to thymectomy before receiving minor lymphocyte stimulating antigen-reactive T lymphocytes. This elimination of lymphocytes without involvement of the thymus implied the participation of a clonal deletion mechanism in peripheral induction of tolerance. In this type of experiment, however, the antigens injected into the animals may have been adequately presented on bone marrow-derived cells with the result that the autoreactive cells could be strongly stimulated within a very short time. In contrast, bone-marrow derived cells would be most unlikely to express tissue specific antigens. On the other hand, Webb *et al.* (1990) also found that the elimination of T cells was not complete. These non-deleted T cells showed only a low level of reactivity to minor lymphocyte stimulating antigens. It is unknown, whether these low reactivity cells were initially a subset of cells with only limited minor lymphocyte stimulating reactivity or whether their low activity was subsequently induced by a "clonal anergy" mechanism.

A "clonal anergy" theory has also been proposed to explain the induction of peripheral tolerance. Schonrich *et al.* (1991) generated two types of transgenic mice. One type of mice was tolerant to the major histocompatibility complex (MHC) class I gene K^b, the other expressed K^b-specific T cell receptor. If these two types of transgenic mice were mated, a marked reduction of clonotype$^+$, CD8$^+$ T cells was observed in the

spleen and lymph nodes of the offspring, whereas, the numbers of Thy-1$^+$, CD3$^-$ lymphocytes were found to be increased. It is unlikely that clonotype$^+$, CD8$^+$ T cells would have been reduced by clonal deletion. Schonrich *et al.* demonstrated that sufficient T cell receptor (TCR) molecules per cell could remain on the surface to allow antigen specific stimulation *in vitro.* Nevertheless, this level of TCR expression was insufficient to be detected by immunofluorescence, leading to the observation of increased frequency of CD3$^-$ cells. They suggested that clonal anergy mechanisms could be responsible for the alteration of TCR.

An immune suppression theory has been advanced as an explanation for peripheral tolerance. Penhale *et al.* (1973) performed thymectomy and then administered low doses of irradiation to neonatal rats. Typical experimental autoimmune thyroiditis developed 6 weeks after surgery. However, this experimental autoimmune thyroiditis could be prevented by transferring thymus-derived cells from syngeneic rats. In this case, autoimmune disease was probably induced by a defect in suppressor cells. This strongly suggested an important role for such cells in peripheral tolerance.

Irrespective of the precise mechanism, the induction of tolerance must require specific reaction between T cells and antigen. The question is that of how T cells recognise peripheral antigens that are only presented on non-lymphoid tissue. Several hypotheses have been proposed to explain this process. The first is that T cells could enter organs such as the pancreas and the ensuing display of self antigens on "non-professional" antigen-presenting cells could lead to anergy (Sprent *et al.* 1992). A second hypothesis is that specific lymphocytes are "tolerized" before they ever meet nominal antigens in the relevant tissue (Coutinho *et al.* 1992). The third suggestion is that peripheral antigens might not be recognised, either because of sequestration in immunologically privileged sites or because of their expression only by cells that could not produce MHC molecules and were therefore unable to present self peptides to T cells. Another possible reason for failure of recognition of a peripherally expressed antigen could be that it was present on the cell membrane at such a low density as to be ignored by specifically reactive T cells (Miller and Heath, 1992).

Both elimination and anergy of immunologically active T and B cells have been reported on a number of occasions. It is likely that "clonal deletion" is the principal mechanism operative in intrathymic tolerance and T cell tolerance, whereas clonal anergy occurs primarily in peripheral and in B cell tolerance. Suppression mechanisms have been implicated in all varieties tolerance.

It is necessary that any attempt to construct a comprehensive explanation of mechanisms of immunological tolerance takes account of the significance of the major histocompatibility complex (MHC). This is likely to be a key factor in the development of self-tolerance. Koller *et al.* (1990) reported the striking result that there was a 100-150 fold reduction in peripheral $CD8^+$ T cells, during the maturation stage of immunocomponent cells, when mice lacking intact β_2-microglobulin genes of MHC class II were produced. This result confirmed that the MHC played an important role in development of T cells and might also be expected to exert a significant effect during the establishment of self-tolerance. The function of MHC molecules could be to collect peptides inside the cell and transport them to the cell surface where they can be recognised by T cells (Rammensee *et al.* 1993). Possible MHC roles in extrathymic tolerance remain controversial. Lo *et al.* (1989) reported that the peripheral expression of I-E on pancreatic cells produced unresponsiveness to I-E by peripheral T cells. It is likely that peripheral tolerance is related to MHC, but peripheral antigens could be expressed only on cells that do not produce MHC molecules.

1.3. Relationship of self tolerance to autoimmunity

Autoimmunity has been explained in different ways reflecting the diversity of theories of self-tolerance. The earliest view, proposed by Ehrlich and Morgenroth (cited by Naparstek and Schwartz, 1988), was that a normal individual must remain unresponsive to self if horror autotoxicus (autoimmunity) is to be avoided. This viewpoint was developed further in Burnet's "clonal deletion" theory. According to Burnet's "clonal deletion" theory, self-tolerance is ensured by the elimination of autoreactive cells, after which none of these cells remain in a normal individual. Autoimmune reactivity, if it appeared subsequently, would result from either the re-

emergence of a "forbidden clone" to an accessible self antigen or the emergence of a new clone reactive against an inaccessible antigen or the generation by somatic mutation of novel antigens (Burnet, 1959).

This explanation has been thrown into doubt by later studies on B cell tolerance. Following the demonstration of a requirement for high doses of antigen for induction of B cell tolerance, Chiller *et al.* (1971) hypothesised that low concentrations of certain antigens in the circulation would only induce T cell unresponsiveness. Cross-reacting or altered antigens could then continue to be recognised as immunogenic by thymus cells and could stimulate bone marrow cells to produce antibody specific for those antigens. Allison (1971) utilised this hypothesis to explain autoimmune thyroiditis. He proposed that low plasma concentrations of thyroglobulin only produce T cell tolerance leaving intact reactive B cells with the function of secreting antibodies. Virus infection, allogeneic antigens, and adjuvants could initiate T cell sensitisation and stimulate these B cells to produce autoantibody leading to thyroiditis.

Following the finding of incomplete, or non-elimination, phenomena in B cell tolerance by Chiller et al. (1971) and Nossal (1975), several investigators demonstrated the retention in normal animals of both T and B cells with autoreactivity. Pereira *et al.* (1985) found that in normal non-immunised mice, more than half of the Lyt-2^+ and L3T4^+ lymphocytes were highly reactive. In contrast with low reactivity cells of the same phenotype, these activated T cells were functional effector cells. L3T4^+ cells could induce proliferation and antibody secretion by normal B lymphocytes. Lyt-2^+ cells efficiently suppressed B lymphocyte responses. These results suggested the existence of autoreactive lymphocytes in normal individuals. Naturally occurring antibodies have also been isolated from normal, unstimulated animals. Guilbert *et al.* (1982) isolated 9 common antigens (tubulin, actin, thyroglobulin, myoglobulin, fetuin, transferrin, albumin, cytochrome c and collagen) from the sera of normal individuals and examined 800 healthy donor sera for antibodies to them. Natural antibodies against all 9 antigens were detected in their donor sera. These data suggested that, regardless of putative clonal deletions, autoreactive lymphocytes remained. In this situation, the maintenance of suppression and of an idiotypic network required reaction between

specific antigen and T cell receptors or interaction among T cell receptors. This type of autoimmune responses has been termed physiological autoimmunity (Coutinho, 1989).

Self-tolerance has been interpreted, according to suppression theories, as an equilibrium between physiological autoimmune responses and auto-reactive cells (Coutinho, 1989). The occurrence of autoimmune diseases might be due to an interruption of, or an aberration in, physiological autoimmunity (Coutinho, 1989). The successful application of T cell vaccination to regulate autoimune diseases has strongly supported this theory. In some animal models of autoimmune disease, e.g. experimental autoimmune encephalomyelitis, and autoimmune diabetes, investigators (Berand *et al.* 1989; Lider *et al.* 1989; Elias, 1991) introduced a dominant, disease-causing clone of lymphocytes into a recipient animal in numbers inadequate to produce disease and observed that this led to protection of the recipient from autoimmune disease caused by future encounters with that clone or with the dominant peptide determinants. Such protection is presumed to be a consequence of the stimulation and expansion of a set of T cell receptor idiotype-recognising cells or T suppressor cells. By extending this explanation, autoimmune diseases could arise through a deficiency or dysfunction in the maintenance or establishment of physiologic autoimmunity.

1.4. Situations in which pathological autoimmune responses have been observed to develop

Pathological autoimmunity might be expected in several situations in human and in animal diseases. Diverse factors could play a part in the development of abnormal autoimmune reactions in each situation. The likeliest situations include abnormalities in structure or distribution of autoantigen, abnormality of the immune system and interruption of normal regulation of autoimmunity as outlined below.

1.4.1. *Abnormal structure or distribution of autoantigens*

A. Release of "hidden antigens"

One of the earliest views was that autoimmune diseases could be induced by exposure of the immune system to sequestered antigens that did not normally contact the immune system during ontogeny and so induce "tolerance". This mechanism of induction of autoimmunity would seem most likely to be important in relation to antigens present within the uveal tissue and the crystalline lens of the eye. Autoantibodies have been repeatedly demonstrated following injuries to those tissues. For example, autoantibodies against sperm and crystalline lens have been detected in serum after vasectomy and eye injury respectively (Fohnson *et al.* 1975; Silverstein, 1975; Anderson, 1967).

B. Exposure of the immune system to modified self-antigens

Pathological autoimmune responses might occur as a consequence of defects in synthesis of self antigens and as an outcome of modifying self antigens by physical, chemical or biological agents, so exposing new epitopes. It has been proposed that viruses possess the capacity to associate with, and convert auto-antigens to, foreign antigens thereby leading to autoimmune responses against them. Wege (1983) used murine coronavirus to induce a subacute demyelinating encephalomyelitis (SDE) in young rats. Neurological symptoms were associated with marked lesions notably primary demyelination in the white matter of the central nervous system. Many rats survived this infection and recovered completely from this central nervous system disease. Among 43 survivors of SDE, 9 rats relapsed 27-153 days after onset of the first attack. Neuropathological examination of these animals revealed areas of fresh demyelination together with old lesions. Viral antigens were detectable in the fresh lesions. In some cases, infectious virus was re-isolated from rats revealing low antibody titres to the virus. In addition, cellular infiltration with lymphocytes, plasma cells and macrophages was observed in damaged tissues. The relapsing, demyelinating disease process, associated with persistent infection and lymphocytic infiltration in target tissues, exhibited some similarities to chronic experimental allergic encephalomyelitis. The occurrence of autoimmune disease in these animals was considered to be a consequence of glycoprotein release from host cell membranes. This was thought to

lead to alteration of antigens on the cell membranes. Inada *et al.* (1984) proposed another possibility. They showed that infection of mice with lactate dehydrogenase virus led to elevation of plasma lactate dehydrogenase, and infiltration of lymphocytes into the central nervous system. They also found that the percentage of cells infected was the same as the percentage expressing antigens encoded by the I region of the MHC. Susceptibility to infection was lost when a rat was treated with purified rat glycoprotein homologous to mouse I-A and I-E antigens. These results strongly suggested that the virus could use MHC molecules as receptors for binding to the cell surface so leading to modification of the presented antigens. Although modification of self-antigens, either by physical methods or by virus, have been hypothesised, studies on *spontaneous* autoimmune diseases have failed to reveal any abnormality in self-antigens (Roitt, 1988). Roitt proposed an explanation for this, noting that neonatal thyroidectomy prevented the spontaneous development of anti-thyroglobulin autoantibodies in Obese strain chickens. However, these animals produced autoantibodies if challenged with thyroglobulin prepared from normal chickens. He suggested that, in this case, the immunological response rather than the antigen was abnormal.

Alteration of autoantigens may be produced by complexing with foreign haptens. One of earliest experimental results on this subject, reported by Ackroyd (1949), was that blood platelets could combine with a drug to form an antigenic complex. The antibodies that developed in response to this antigen were highly specific for the complex. An outstanding clinical example of this phenomenon is the thrombocytopenic purpura that has developed in some individuals under treatment with the hypnotic sedormid (allyl-isopropyl-acetyl carbamide) (Ackroyd, 1949).

C. Exposure of the immune system to cross-reactive antigens

Specific immunological self-tolerance can be terminated by immunisation of an animal with other antigens that cross-react with the tolerated antigen. In human rheumatic fever, antibodies produced in patients to the *streptococcus* also reacted with heart muscle (Krisher *et al.* 1985; Dale *et al.* 1982). Dale *et al.* (1982) immunised

rabbits with the proteins of group A *streptococcus* and evoked an antibody that cross-reacted with the sarcolemmal membrane of human heart muscle cells. Krisher *et al.* (1985) further demonstrated that rabbit skeletal muscle myosin and human heart extract reacted with a monoclonal antibody that also reacted with *Streptococcus pyogenes.* This result strongly suggests that heart myosin shares immune determinants with a component of *S. pyogenes* as a result of which an immune response against heart myosin could be initiated.

1.4.2. *Abnormality of the immune system*

According to the "clonal deletion" theory, autoimmune disease could be expected to occur following mutation of self-antigens or entry of cross-reacting foreign antigens into the body. Hence, modification of antigens or mimicking of antigens have been emphasised as likely precursors to autoimmune disease. Indeed, during pathological autoimmune responses in human patients, various disturbances of immunologically component cells appeared to be restricted to specific antigens. Sun *et al.* (1991) reported that the number of autoreactive T and B cells specific for proteolipin protein were increased in the peripheral blood and in the cerebrospinal fluid of patients with multiple sclerosis. It is likely that alteration to a specific proteolipin antigen initiated the increase in numbers of specifically reactive lymphocytes. However, lymphocytes reactive against several other antigens have also been reported in patients with the same disease (Sun *et al.* 1991).

Additionally, Klinman (1990) reported that polyclonal B cell activation in lupus-prone mice preceded the development of autoimmune diseases. He examined a group of lupus-prone mice that exhibited a polyclonal increase in numbers of Ig-secretory spleen cells at 10 weeks of age. The disease was characterised by the production of IgG antibodies reactive with a variety of self-antigens, and led to glomerulonephritis at 5-9 months of age. The increase of polyclonally activated B cells was obvious earlier and was likely to be causally related to the occurrence of autoimmune disease. He then proposed that polyclonal activation of B cells might

initiate the production of those autoantibodies that caused tissue damage and the release of autoantigens.

In recent years, genetic studies of the role of non-MHC-linked genes in autoimmune diseases have revealed the involvement of the TCR β-chain locus in autoimmune diseases. For example, Frank *et al.* (1990) reported that specific anti-Ro autoantibodies were often associated with the T cell receptor Cβ1, Cβ2 locus in systemic lupus erythematosus patients. This association occurred in 76% of patients with Ro precipitins. The high frequency of association between anti-Ro antibodies in SLE patients and a part of the TCR β locus suggested that a pair of TCR β genes were not disease susceptibility markers, but rather were involved in the production of anti-Ro antibodies. This result strongly suggested an important role for preceding abnormalities of immune cells in the occurrence of autoimmune diseases.

1.4.3. *Interruption of normal regulation of autoimmunity*

A. Escape from self-tolerance

Escape from self tolerance is likely to be associated with the appearance of autoimmunity. Amagai (1981) studied an SLE susceptible strain of mice and found that T cells in these mice were resistant to the induction of tolerance to heterologous γ-globulins. Amagai found that these mice developed resistance to the induction of tolerance to rabbit γ-globulin from the early age of 10 weeks. These mice also had T cell defects. He suggested that defects of T cells in these mice might be attributed either to loss of suppressor T cell activity and/or to some form of interference with central T cell tolerance in T helper cells. Resistance to tolerance could be a result of a thymic defect. Theofilopoulos *et al.* (1981) reported that all strains of mice that were prone to develop systemic lupus erythematosus (SLE) also developed early thymic atrophy, which particularly involved the cortex. In addition, all SLE-prone strains of mice manifested a premature decline (as early as 1 month of age) in the production of a circulating thymic hormone, thymulin (Bach *et al.* 1979). The thymus and its hormone are essential for the differentiation of T cells and their helper, suppressor and cytotoxic

subsets. Deficiency of thymulin would be expected to influence the development and function of T or B cells.

B. Defect of suppressor cells

The thymic defect and the resistance to induction of tolerance in SLE-prone strains of mice could lead to defects of suppressor T cell activity. Krakauer *et al.* (1976, 1977) observed decreased numbers and dysfunction of Ts cells in NZB/W F_1 mice. Decreased numbers and activity of Ts cells have been commonly reported in patients with SLE (Miller *et al.* 1979; Morimoto, 1980). However, there are also some contrary findings. Creighton *et al.* (1979) reported that no significant difference was observed between autoimmune mice and normal control strains either in the antibody-producing abilities of specific B lymphocytes or in the regulatory function of antigen-specific helper and suppressor T cells. This finding applied even in those circumstances in which intact mice showed a marked deficiency in antibodies.

C. Disturbance of idiotypic interactions

Although the subject remains controversial, the involvement of idiotypic networks in the pathogenesis of autoimmune disorders has been reported. Shoenfeld *et al.* (1990) showed that SLE could be experimentally induced in naive mice after immunization with the human 16/6 idiotype which was a representative idiotype of anti-DNA autoantibodies. An SLE-like disease in these animals was characterized by clinical (proteinuria), serological (anti-dsDNA, anti-Sm antibodies), and pathological findings. The disease could be interrupted by treatment with T suppressor cells that had been generated specifically against the pathogenic 16/6 idiotype of anti-DNA autoantibodies. They interpreted these results as indicating that in the SLE patient, a defect in T regulatory cells might lead to the emergence of IgG idiotypes or of antibodies with high affinity with a pathogenic role. In other words, autoimmune disease could be induced by dysregulation of a functional network of idiotype-anti-idiotype interactions among auto-reactive B cells, T helper cells, and T suppressor cells.

D. Deviation of MHC

The expression of MHC class II antigens on the surface of non-lymphoid cells, e.g. thyroid cells, has been reported by Pujol-Borrell *et al.* (1983) in human autoimmune thyroiditis (Graves' diseases, GD; Hashimoto's diseases, HD) . This has been interpreted as a primary cause in the induction of those diseases (Bottazzo, 1983). However, recent experimental data suggested that the expression of class II antigens on thyroid epithelial cells (TEC) could be the result of release of interferon-γ (IFN-γ) by adjacent lymphocytes (Hamilton *et al.* 1991). The question of whether the expression of MHC on TEC is a primary or secondary event in the development of autoimmune thyroiditis. will be considered further in Chapter 1.6.

A genetic association between MHC and autoimmune diseases has been demonstrated (review by de Vries *et al.* 1988). The mechanism of the association is unclear. One possibility is that antigen binds more easily to the MHC molecules of individuals with some specific MHC haplotypes on their accessory cells. Although direct evidence in support of this hypothesis is lacking, a report from Osoba & Falk (1978) has indirectly supported it. They found an increased mixed leucocyte reactivity in individuals with an human leucocyte antigen-B8 (HLA-B8) phenotype, both normal individuals and patients with untreated Hodgkin's diseases. The similarity of results in healthy individuals with HLA-B8 phenotype to those in patients with Hodgkin's disease with HLA-B8 suggest that increased mixed leucocyte reactivity is not an effect of the disease process. It could be due to sensitive interaction between antigen-MHC accessory cells and T cell receptors.

E. Influence of genetic factors

Potential genetic contributions to autoimmune diseases have been assessed by following disease expression in families and by comparing the concordance rate in monozygotic (identical) versus dizygotic (non-identical) twins. Block *et al.* (1975) examined 12 sets of twins, seven of them definitely monozygotic and another three definitely dizygotic. They found that four of seven pairs (57%) of definitely monozygotic pairs were concordant for SLE , including clinical phenomena that satisfy

the preliminary criteria of the American Rheumatism Association, namely the presence of antinuclear factor and hypergammaglobulinemia. All three of the dizygotic sets of twins were discordant for clinical SLE, although one clinically well twin had marked serologic abnormalities. The high concordance rate in monozygotic twins suggested that genetic factors were significant in the pathogenesis of SLE. However, more recent work produced a significantly different result. Deapen *et al.* (1992) used deoxyribonucleic acid (DNA) fingerprinting to validate the reported zygosity in a sample of these twins. By examining 107 twin pairs meeting the American College of Rheumatology (1982) revised criteria for the diagnosis of SLE, they found 24% of monozygotic pairs and 2% of dizygotic pairs were concordant. The higher concordance rates in monozygotic than in dizygotic twins supported the operation of inherited determinants in the pathogenesis of autoimmune diseases. However, the concordance rate of monozygotic twins in this report was lower than that in the previous report. They deduced that immune competence and immune susceptibility in humans were generated by a stochastic recombination of gene fragments at the time when precursor cells were developing into mature T or B lymphocytes. They also suggested a possible role for enviromental determinants in SLE expression.

On the assumption that a genetic factor is implicated in autoimmune diseases, genes associated with the disease have been widely examined. Associations of genetic factors with human autoimmune diseases are clearly evident (de Vrise, 1988). Rheumatoid arthritis correlates chiefly with possession of DR_4 and systemic lupus erythematosus is now generally associated with DR_2 and DR_3. Graves' and Hashimoto's diseases are closely related to DR_3 and DR_5 respectively. In recent years, genes encoding the T cell receptor have attracted much attention. Hirose *et al.* (1991) analysed the involvement of the TCR β chain locus derived from the NZW strain in the severe lupus-like disease occurring in the (NZB×NZW)F_1 hybrid. They found that a gene within, or closely linked to, TCR β on chromosome 6 of NZW mice was associated with increased production of autoantibodies to histone and double- and single- stranded DNA and with accelerated renal disease. Although mechanisms whereby genetic predispositions to autoimmune disease are realized remain unclear,

several possibilities have been proposed. According to one, autoimmune disease might arise through cross-reactivity between MHC molecules of patients and bacterial antigens. van Bohemon *et al.* (1984) showed that monoclonal anti-HLA-B27 antibodies also reacted with bacteria, (e.g. *Klebsiella, Shigella,* or *Yersinia*), which were considered to be pathogens of Reiter's disease. These results support the hypothesis that molecular similarity between HLA-B27 and certain bacterial antigens might result in autoimmune diseases. The evidence for this hypothesis is, however, still far from convincing, because it fails to explain the actual process causing the disease. On the other hand, MHC association with susceptibility to autoimmune disease might reflect, not a direct role of MHC molecules in disease pathogenesis, but rather a role of products of other genes that exist in linkage disequilibrium as part of more extended MHC haplotypes. de Vries *et al.* (1988) showed that deficiency of the second factor of complement (C2) and the C4A-null alleles which were a part of extended MHC haplotypes were associated with SLE. They assumed that the alteration of C4A-null alleles could influence the normal clearance of immune complexes. A third mechanism, by means of which specific genetic predisposition to autoimmunity could be achieved, might involve the direct influence of T cell receptor genes on the function of T cells, mediated either by susceptibility to autoimmune disease (Osoba & Falk, 1978) or by the production of specific autoantibodies such as anti-Ro antibodies (Frank *et al.* 1990).

1.5. Experimental animal models for the investigation of autoimmune responses

Animals are a valuable source of materials whereby pathogenic and regulation mechanisms could be accessible to study. Investigators have long searched for a suitable animal model to study autoimmune responses. In 1903, Uhlenhuth (cited by Burnet, 1972) showed that lens protein from rabbits could produce precipitins in the same species. The introduction of Freund's complete adjuvant greatly increased possibilities for experimental autoimmunization. Since then, various animal models of autoimmune disease have been produced. Most studies on autoimmune reactions have been based on 5 types of experimental autoimmune animal models. To summarize these, autoimmune responses have been induced by immunisation with extracts of a specific organ plus

adjuvant or by neonatal thymectomy. Autoimmune responses can be predictably observed in some strains of animal selected on a genetic basis. Autoimmunity may also arise in transgenic animals expressing neoantigens or as a consequence of organ ablation before immunological maturation.

1.5.1 *Experimental autoimmune responses induced by immunisation with extracts of specific organs plus adjuvant*

In 1903, Uhlenhuth (cited by Burnet 1972) used Freund's complete adjuvant (FCA) to increase the likelihood of immune response induction in rabbits. Subsequently, this method has been widely utilised to produce specific immune responses to corresponding antigens from the same or a not too distantly related species. For example, thyroiditis appeared in rabbits after injection of thyroglobulin (Tg) associated with adjuvant (Witebsky *et al.* 1956). Experimental encephalomyelitis could be induced in a variety of species by the injection of central nervous tissue with adjuvant (Arnon, 1981). The autoimmune nature of diseases experimentally induced in those animals have been confirmed by the presence of auto-antibodies to target antigens, and infiltration of the damaged target tissue by mononuclear cells. These immune responses could usually be transferred to syngeneic recipient animals by lymphoid cells from immunised donors and occasionally by serum (Burnet, 1972).

In this type of model, adjuvants appeared to exert an overt influence on the occurrence of autoimmune diseases, because their use clearly increased the likelihood of appearance of autoimmune responses and increased the titre of auto-antibodies. The mechanism of adjuvant action has been proposed to entail the slow release of soluble antigen into the general circulation. Antigens in the granuloma formed after injection of adjuvant might be carried by immune cells entering the granuloma, subsequently leaving it by lymphatic channels to enter draining lymph nodes or to reach the spleen and other lymphoid tissues via the blood (Burnet, 1972).

It has, nevertheless, been shown that even a rabbit's own thyroid tissue, obtained by surgical removal of half of the gland, could be used as an antigen, together with adjuvant, to produce a dense, small cell infiltration in the remaining thyroid tissue. In

this instance it was not necessary to use tissue from a different species to evoke autoimmunity (Burnet, 1972). This phenomenon raises a query about the role of adjuvant in induction of immune responses in experimental animals. Kleinan (1991) reported that an oily adjuvant could induce arthritis in the DA rat. In this case, the arthritis was associated with the infiltration of mononuclear cells, suggesting the involvement of immunologically mediated inflammation in the joints. This implied that adjuvant has a role as an immunologic trigger, in addition to any physical, accessory effects. Rook *et al.* (1991) also found that administration of an oily adjuvant triggered increased secretion of interleukin-6 from synovial cells. Interleukin-6 has been thought to have B cell-regulatory properties and to be able to enhance the activity of B cells. Hence, the cellular and humoral responses in this type of model could be induced, at least partly, by the adjuvant itself. Indeed, the auto-antibodies in some examples of this type of experimental autoimmune thyroiditis (EAT) model were polyclonal with capacity to recognise approximately 40 different epitopes. This contrasts with the antibodies from patients with Hashimoto's diseases which are reported to recognise a more restricted number of determinants (Charreire, 1989).

1.5.2. *Autoimmune reactivity induced by neonatal thymectomy*

The first experiments in this category were undertaken by Penhale *et al.* (1973), who showed that neonatal thymectomy in the rat, followed by several low doses of irradiation, led to the spontaneous development of typical EAT without any requirement for Tg injection. These models were characterized by increased titres of anti-thyroglobulin antibodies, and overt infiltration of mononuclear cells into thyroid tissue. Furthemore, they demonstrated that this form of EAT could be prevented by reconstitution of these animals with viable lymphoid cells from syngeneic rats (Penhale *et al.* 1976). The depletion of specific suppressor cells as a result of thymectomy was considered to be responsible for the autoimmune responses.

1.5.3. *Spontaneous autoimmune responses determined genetically*

In 1959, Bielschowsky (1970) developed the New Zealand Black (NZB) strain of mouse which, as it aged, consistently developed an autoimmune haemolytic anaemia with positive Coombs test. This outcome could also be provoked in young, otherwise unaffected, NZB mice following the transfer of spleen cells from a Coombs positive donor. These mice, and especially their F_1 hybrids with the partially related New Zealand White strain, (NZBxNZW)F_1, had a high incidence rate of various circulating autoantibodies and antoimmune-complex-induced glomerulonephritis (Warner, 1977). Since then, several other strains of animals which spontaneously develop different types of autoimmune diseases have been discovered. For example, autoimmune thyroiditis occurred in Obese strain chickens and in Buffalo rats (Khoury *et al.* 1982; Schanenstein *et al.* 1985). These strains of animals were characterised by spontaneously occurring, familial autoimmune diseases. This characteristic strongly suggested that genetic factors control their induction. Burnet (1972) proposed that there might be a genetically based functional abnormality as a result of which NZB mice were hyper-responsive to antigens and less readily developed tolerance to any antigen. Many genes have subsequently been revealed to be related to the occurrence of autoimmune diseases. Genetic studies have provided information about the identity of genes possibly responsible for autoimmune diseases. However, the mechanisms by which genes responsible for autoimmune disease achieve their effects remain undetermined.

1.5.4. *Spontaneous autoimmune responses in transgenic animals*

Recently, a number of transgenic animal models have been developed for study of autoimmune responses. The design of these models has started with the premise that tolerance to self is an active process, in which encounter of the immune system with autoantigens during foetal life leads to self-tolerance. Any exoantigen expressed during this stage should be tolerated thereafter by the recipient immune system. Transferring a specific gene to an embryo can produce a transgenic mouse tolerant to the product of that gene. However, in later life, the transgenic animal may develop an autoimmune disease as a result of possessing that gene. Okamoto *et al.* (1992) produced transgenic mice bearing Ig heavy and light chain genes encoding an autoantibody against mouse

erythrocytes. They observed that 50% of these transgenic mice had varying degrees of autoimmune hemolytic anaemia. Transgenic models have been widely utilised to explore the mechanism of either self tolerance or autoimmunity.

1.5.5. *Autoimmune responses induced by organ ablation*

The approach of using organ ablation to induce autoimmune disease is based on the hypothesis that if a foreign antigen implanted during foetal life can be tolerated by the recipient, then the removal of an auto-antigen during foetal life should render the animal capable of mounting immune responses against re-implanted self-antigens in adult life. Triplett (1962) removed the buccal component of the tree frog pituitary gland at the larval stage, maintained it in another larval tree frog for 2 months, and then re-implanted it into the metamorphosed owner. Acceptance or rejection of the reimplanted tissue was assessed functionally by skin colour changes, because the hypophysis secretes melanophore stimulating hormone (MSH). Ten out of 13 of the replaced glands were found to have been rejected. To exclude the possibility that these immune responses had been induced by contaminating tissue from the intermediate host, 7 tree frogs were submitted to removal and subsequent re-implantation of only half of the posterior pituitary gland. In all cases, frogs which had had only half of the hypophysis removed accepted this tissue when it was reimplanted.

Experiments were also done to determine whether frogs of the stage of development at which pituitary tissue had been re-implanted had acquired the capacity to reject pituitary allografts. Six out of 9 allografts implanted in hypophysectomized recipients were rejected but the remaining 3 were accepted, perhaps suggesting that the latter animals remained immunologically immature at the time of re-implantation. Triplett's experimental results suggested that interference with self-tolerance by means of self-tissue removal before self-recognition had been acquired could produce an autoimmune response against re-implanted auto-tissue after the establishment stage of self-tolerance.

A contrasting result was obtained Rollins-Smith and Cohen (1982) who employed an experimental model similar to that of Triplett to reinvestigate the timing of

development of immunological tolerance to self-organ specific-antigens. They removed both pituitary and eye anlagen from frog embryos at a time similar to that at which Triplett had intervened. All self-grafts of eye anlagen and pituitary anlagen were successfully accepted after re-implantation. Allogeneic eyes were rejected, but only by about half of the intact larval hosts tested. Six out of 9 recipients of allogeneic pituitary gland implants rejected these tissues suggesting that not all frogs were immunologically mature by the time of re-implantation.

Rollins-Smith and Cohen proposed several possibilities to explain their failure to reproduce Triplett's finding of frequent rejection of re-implanted self tissues. The first was that , as different strains of tree frogs had been used by the two groups of investigators, strain specific differences in the rate of maturation of immunity were possible. The second possibility was that differences in the sites of placement of the pituitary anlagen in the two experiments could have influenced the outcome. In the Rollins-Smith experiments, the tissue was "parked" in previously hypophysectomized age-matched sibling embryos. In contrast, Triplett parked the tissue under the ectoderm in the tail region. The formation of intermediate host-derived fibrous connective tissue in the reimplanted hypophyseal tissue could have stimulated an immune response against it by the final recipient. A third possibility could be that some frogs retain a capacity to become tolerant to self-organ-specific antigens throughout life. A fourth possible explanation could be that tissue-specific antigens capable of recognition by the immune system were not present in Rollins' models.

Recently, Eishi and McCullagh (1988) and McCullagh (1989) described two animal models in which autoimmune responses could be induced by interference with self-recognition during foetal life in rats or in lambs. Thyroid tissue has been used as a target tissue. After surgical removal of the thyroid, or interference with thyroid development by ^{131}I during foetal life, autoimmune responses were initiated in response to encounter with self or syngeneic thyroid in later life.

In the foetal lamb model, in which the thyroid gland was surgically removed one third of the way through gestation, severe infiltration by lymphocytes was observed at autopsy in reimplanted autologous thyroid tissue in all 8 cases tested (McCullagh,

1989). To exclude the possibility that these immune responses in the foetal lambs had been initiated by contaminating cells from the intermediate nude mouse host, hemi-thyroid removal and reimplantation was performed in foetal lambs. In all 5 such cases, reimplanted thyroid tissue remained free from lymphocyte infiltration.

In DA rats, the thyroid glands of which had been destroyed by administration of ^{131}I on the seventeenth day of gestation, 40 out of 59 cases were characterized by lymphocytic infiltration in reimplanted tissues (Eishi and McCullagh, 1988). The control procedure of implanting syngeneic thyroid in normal rats was employed to exclude the *de novo* induction of immune responses by syngeneic antigens. In all 17 control rats, implanted syngeneic thyroid remained completely free from lymphocytic infiltration. As additional control procedures, syngeneic parathyroid, pituitary and adrenal tissue were implanted, together with thyroid tissue, into syngeneic rats previously treated with ^{131}I. Whilst inflammation was present in the accompanying thyroid graft in 7 recipients, all other grafted tissues remained totally free from lymphocyte infiltration. These results suggested that the autoimmune response was induced by interference with tolerance of immune cells to thyroid antigens. They could not be explained in terms of a reaction against contaminating cells derived from an intermediate host or of a reaction newly induced against syngeneic antigen itself.

The time at which the foetal lamb's thyroid was removed (54-55 days gestation) was selected on the basis of an earlier observation that 54 days foetal lambs would accept allografts of adult skin (McCullagh, 1988). This indicated that the immune system had not become competent.

Lymphocytic infiltration was also found in autologous thyroid tissue reimplanted in 2 foetal lambs which retained small residual thyroid remnants in their cervical region. This suggested that a certain level of self antigen determinant expression was required to ensure the development of self tolerance. Expression of antigen below this threshold failed to provide sufficient tolerogenic stimulus. Similar residual thyroid tissue showing marked atrophy, remained in rats that manifested thyroid autoimmunity as a result of exposure to ^{131}I *in utero*.

Selection of the thyroid gland as the target organ in these "organ ablation" experiments could raise the question of whether thyroglobulin was a "forbidden antigen". Triplett (1962) decided not to use the thyroid gland in his original experiments on the basis of this reservation. The rationale was that animals would be expected to retain the capacity to react against a forbidden antigen, irrespective of whether the organ producing it had been extirpated before immunological competence had been acquired or left intact. However, more recently, the presence of thyroglobulin in lymphatics and serum has been recognised. Daniel *et al.* (1966) confirmed its presence in lymph draining from the thyroid, and subsequently quantitated the extent of its release into the lymphatics. Torrigiani *et al.* (1969) reported that 60-70% of normal human subjects had demonstrable thyroglobulin in the circulation. On the other hand, the complete absence of anti-thyroglobulin antibody from the serum of rats exposed to ^{131}I in foetal life implies that thyroglobulin is not the target antigen in this model (Eishi and McCullagh, 1988).

Another question raised by these experiments is that of whether the observed immune responses were induced directly by absence of thyroid self-determinants or were initiated only by the re-introduction of these antigens following the implantation of self thyroid tissue into recipients with a mature immune system. Interpretation of the response to reimplantation must take account of the influence of thyroid hormone on development of the immune system. The effects of thyroid hormones on the immune system are incompletely understood. Fabris (1973) reported that the removal of the thyroid gland from rats reduced the peripheral blood level of lymphocytes, depressed antibody responses and lowered the responses of spleen cells to mitogens. The administration of thyroxine reversed these effects. These results indicated the positive effect of thyroid hormones on the immune system's function. In other words, the removal of the thyroid gland could be expected to depress immune responsiveness non-specifically, but not to enhance it.

Models of autoimmunity following organ ablation provide an opportunity to study regulation of autoimmunity. McCullagh (1990) surgically parabiosed DA rats, that had been exposed to ^{131}I in foetal life, with normal syngeneic rats. Syngeneic

thyroid lobes were introduced under the renal capsule of each parabiont. No instance of thyroiditis was observed in implants in either the ^{131}I-exposed or normal partner, if these were placed simultaneously with, or after, parabiosis. However, thyroiditis was evident in implants in ^{131}I-exposed rats, parabiosis of which had been deferred for 1-2 weeks after graft placement. In addition, marked thyroiditis commonly developed in thyroid implants in both parabionts, if the normal parabiont had received irradiation before surgery. This result suggested that normal DA rats possess migratory, radiosensitive cells with the capacity to curtail any expression of anti-thyroid reactivity by rats in which thyroid development has been disrupted before the development of immunocompetence.

1.6. Investigation of the regulation of autoimmunity

The majority of experimental investigations of the pathogenesis of autoimmune disease have been concerned with the re-emergence or re-activation of auto-reactive cells and the modification or mimicking of self-antigen. Less investigation has been directed to mechanisms of regulation of autoimmunity. This tendency may reflect the major emphasis placed on clonal deletion and clonal anergy hypotheses to explain self-tolerance. The appearance of autoreactive cells in overt autoimmunity is more amenable to investigation than are the reasons for their absence from, or poor detectability in, normal self-tolerant animals. Nevertheless, as already noted, potentially autoreactive cells have been detected in normal animals and patients. This has re-directed attention to the possibility of negative regulation, or suppression, of autoimmune processes in normal individuals.

The existence of suppression mechanisms in some forms of artificially induced immunological tolerance has been recognised for a considerable time. McCullagh (1970a,b) reported that the transfer of thoracic duct lymphocytes from normal syngeneic donors failed to initiate a haemolytic antibody response in rats tolerant of sheep erythrocytes. Rats inoculated with sheep erythrocytes on the day of birth and then twice weekly for a further 10 weeks became immunologically tolerant of sheep erythrocytes in that no haemolysin could be detected even at the lowest serum dilution.

If these rats received thoracic duct lymphocytes from normal, syngeneic rats, no antibody response was detected, reflecting specific suppression of the relevant immunological reactivity of the transferred cells.

Subsequently, Gershon *et al.* (1971) transferred splenic cells from mice made tolerant to sheep red blood cells to normal syngenic mice. They also found the prevention of immune responses to sheep red blood cell in the normal syngeneic recipients. They concluded that bone marrow cells were unlikely to have been responsible for this suppressive effect, because suppression occurred without production of any significant amount of detectable antibody. Subsequent experiments (Gershon *et al.* 1973) showed that the suppression effect was mainly attributable to thymus-processed lymphocytes.

Whilst the significance of suppression or negative regulation of immune responses as the basis for artificially induced immunological tolerance remains uncertain, its biological relevance for self tolerance in normal individuals remains even less clear. In some instances, individuals subjected to an autoimmune disease recovered from it, or became resistant to further attacks (Swanborg and Welch, 1978). Swanborg and Welch (1978) showed that the occurrence of spontaneous recovery from experimental allergic encephalomyelitis could occur, even in susceptible rats. Rats that had recovered from experimental autoimmune encephalomyelitis (EAE) appeared to be resistant to further attempts to initiate EAE. Suppressor cells, able to interfere with the occurrence of EAE, have been demonstrated in these rats.

The increased frequency of autoimmune thyroiditis following neonatal thymectomy and its reduction following transfer of T lymphocytes, supports the contention that suppression is important as a mechanism to prevent induction of autoimmune diseases in normal animals (Penhale,1973).

Autoimmune disease could arise through the lack of specific idiotypic regulation. Howell *et al.* (1989) vaccinated Lewis rats with synthetic peptides corresponding to idiotypic determinants of the β chain VDJ region of the T cell receptor that were conserved among encephalitogenic T cells. These rats were thereby rendered resistant to the induction of EAE. This finding suggested that lack of specific idiotypic

control could be responsible for the induction of autoimmune diseases and as a corollary, that the stimulation of specific anti-idiotypic reactivity by vaccination against idiotypic determinants of a pathogenic T cell receptor could modulate autoimmune diseases.

However, despite extensive research, immunologists still have not been able to prove unequivocally that Ts cells exist as a discrete entity (Moller, 1988). Moller summarised the main doubtful points as follows, (1) No unique surface markers for Ts cells have been demonstrated. (2) The I-J gene believed to govern Ts-cell function has not been mapped in the MHC. (3) TCR genes did not function in Ts cells. Uncertainty about the existence of T suppressor cells effectively raises doubts about the relevance of T suppressor effects for regulation of autoimmune reactions. In addition, there are some models of autoimmunity in which no abnormalities of Ts-cells can be detected. (Feasby *et al.* 1984). Despite the abundant data showing Ts-cell defects in autoimmune conditions, the pathogenesis of these defects remains uncertain as does the question of whether they represent the primary event in the induction of the autoimmune condition or only a consequence of an earlier aberration.

1.7. Specific features of thyroid autoimmunity

As the thyroid is the target tissue selected for the investigation of autoimmunity in this thesis, experimental investigation and specific features of autoimmune thyroiditis will be briefly reviewed. Thyroid specific antigens appear to be peripheral antigens that are unlikely to be expressed in the thymus (Lo, 1992). Consequently, it is likely that some features of thyroid autoimmunity may be shared in common with autoimmunity to other peripheral antigens.

1.7.1. *Pathogenic mechanisms of autoimmune thyroiditis*

Several pathogenic mechanisms have been proposed for autoimmune thyroiditis. In 1971, Chiller *et al.* found that the injection of low doses of bovine serum albumin or human gamma globulin into mice induced unresponsiveness selectively in T lymphocytes, leaving the response of B lymphocytes unimpaired. In contrast, high

doses of these antigens induced unresponsiveness in *both* T and B lymphocytes. Even with the administration of very high doses of antigen, some B lymphocytes remained responsive. On the basis of this result, Allison (1976) proposed that antigens, such as thyroglobulin which were present at low concentration in the circulation only induced T cell tolerance, leaving B cells able to respond to them. If the corresponding antigen-specific T cells were stimulated by a cross-reacting antigen, autoantibodies produced by B cells against thyroid specific self-antigens would be induced. He suggested that the introduction of adjuvant and the administration of heterologous thyroglobulin possessing determinants cross-reactive with recipient thyroglobulin, could directly stimulate B cells. In addition, he hypothesised that virus infection could bypass T cell stimulation to initiate B cell responses directly.

Subsequently, a striking finding reported by Penhale *et al.* (1973) was that thyroiditis could be evoked in rats by a combination of thymectomy and irradiation. Sixty percent of Wistar rats prepared in this manner were found to have severe infiltration of lymphocytes in their thyroid tissue and an increased level of anti-thyroglobulin antibody in their serum. Antinuclear antibodies were also found in approximately 10% of these animals. Plasma cells appeared to be more prominent in some animals. In interpreting these results, it was suggested that, under normal circumstances, B cells autoreactive with thyroid antigens were prohibited from expressing reactivity by the presence of thymus-derived lymphocytes. It was inferred that the removal of T lymphocytes by thymectomy and irradiation led to B cell clonal expansion and subsequent thyroiditis. This hypothesis has been supported by subsequent experiments in that, when syngeneic lymphocytes have been injected into rats already subjected to thymectomy and irradiation, autoimmune thyroiditis is prevented (Penhale *et al.* 1976). These results strongly imply the existence of a defect of T suppression as a contributing factor in the induction of autoimmune diseases with B cell activity following as a consequence.

In 1983, Pujol-Borrell *et al.* reported the expression of MHC class II antigen on thyrocytes from patients with two manifestations of human autoimmune thyroiditis, namely Hashimoto's and Graves' diseases. Pujol-Borrell, *et al.* cultured thyroid cells

from these patients with phytohemagglutinin and then examined them by means of indirect immunofluorescence staining. The positive expression of MHC class II antigen detected in these patients, did not occur in normal human thyrocytes. In the light of these results, Bottazzo *et al.* (1983) proposed that class II antigen expressed on non-lymphocytes enhanced the presentation of autoantigen on the cell surface. It has been suggested that these antigens usually appear on the cell surface at too low a density to induce tolerance, but that if they are presented by MHC class II molecules, stimulation of T cells could result. Bottazzo *et al.* also suggested that viral infection with secretion of IFN-γ induced the expression of MHC class II antigen, because IFN has been reported to induce class II expression on epithelial cells (Steeg *et al.* 1982)

A contrary viewpoint has been presented recently by Hamilton *et al.* (1991), namely that aberrant class II MHC expression by thyroid epithelium cells in established thyroid autoimmune disease could be the *result* of release of interferon-gamma (IFN-γ) by adjacent lymphocytes. They examined the expression of class II antigen and IFN in thyroid tissue from a group of Hashimoto's and Graves' disease patients. They found that thyrocyte class II MHC expression in these cases usually occurred adjacent to IFN positive staining lymphocytes. Both expression of class II antigen and positive staining for IFN appeared in areas containing a larger number of infiltrating lymphocytes. They explained their results on the basis that lymphocytes within the thyroid could result in release of IFN and subsequent expression of class II MHC antigen on thyroid cells.

1.7.2. *Cell-mediated immune responses in autoimmune thyroiditis*

Autoimmune thyroiditis is manifested, both in humans and in animals, by diffuse infiltration of the target tissue with lymphocytes. Infiltrating lymphocytes may aggregate to form secondary follicles or germinal centres. It is unclear whether the majority of infiltrating lymphocytes are T cells cytotoxic for target cells, or B cells that could produce antibodies against the target cells.

Canonica *et al.* (1985) analysed subpopulations of infiltrating lymphocytes in thyroid tissue from a group of patients with Hashimoto's disease. They found that 50% of the infiltrating T lymphocytes were $CD8^+$ in 4 out of 6 cases tested. These $CD8^+$

cells proliferated when they encountered human thyroglobulin antigens. They explained that these activated $CD8^+$ cells included cytotoxic $CD8^+$ cells which could cause cytolytic effects. Most and Wick (1986) examined the localisation, phenotype and distribution of lymphocytes in thyroid tissue sections from Hashimoto's disease patients. They found that the majority of cells infiltrating the thyroid tissue were T cells, but that $CD4^+$ and $CD8^+$ cells differed in their tissue distribution. In the interstitium, $CD4^+$ cells were the most abundant, whereas in relation to peripolesis within thyrocytes, $CD8^+$ cells were much more common. This implied that the $CD8^+$ cells could play a role in thyroid tissue destruction.

Using experimental animals, Creemers *et al.* (1983) showed that lymph node cells from mice immunised with mouse thyroglobulin initiated cytotoxic responses against thyroid monolayers and that the effector cells were thymus derived. In contrast, there have been some reports of B cell produced antibodies mediating autoimmune thyroiditis. Jansson *et al.* (1983) reported that there was a higher percentage of B cells than of T cells within the thyroid of Hashimoto's thyroiditis patients. These data were in accord with the high rate of local synthesis of thyroid-directed antibodies that was observed. The higher percentage of B cell and local synthesis of thyroid-directed autoantibody suggested the occurrence of a humoral mediated immune response against the thyroid cells.

1.7.3. *Humoral immune responses in autoimmune thyroiditis*

The production of auto-antibodies against thyroid antigens is a common feature and is readily detected in autoimmune thyroiditis. However, the significance of humoral responses in the pathogenesis of thyroiditis is a much more controversial issue. Evidence is available to support a range of opinions.

Although autoantibodies against thyroid antigens are readily detected in the plasma of individuals with autoimmune thyroiditis, there is frequently a lack of correlation between circulating autoantibodies and the severity of the diseases. McMaster *et al.* (1961) immunised guinea pigs with adjuvant plus thyroid extracts derived from the same strain or other strains of guinea pig. They found a lack of

correlation between auto-antibody titres and lesions in the thyroid. Thirty-two guinea pigs did not have detectable antibodies 5 days after immunisation, whereas 5 of these animals had already developed thyroiditis. By contrast, 21 other guinea pigs with detectable antibodies, albeit of low titre, did not have thyroiditis when examined 7 weeks after immunisation. They emphasised that these experiments failed to demonstrate a role for antibodies in the aetology of EAT.

Penhale *et al.* (1975) also found that in some strains of rat, moderate titres of antibody developed without concomitant thyroid damage. To explain this observation they suggested that a certain threshold level of autoantibody would be required to overwhelm the regulatory mechanism and initiate thyroid damage. The expression of overt thyroid autoimmunity within a particular strain would then depend upon the loss of equilibrium between autoantibody production and some form of local suppressive activity.

Other researchers have reported good correlations between anti-thyroglobulin antibody titres and the severity of thyroiditis. Clinton *et al.* (1972) examined thyroid glands from NZW mice immunised with heterologous thyroglobulin for anti-thyroglobulin antibodies. There was a direct correlation between the appearance of anti-thyroglobulin antibodies in the thyroid gland and the appearance of thyroid lesions, which were not observed before anti-thyroglobulin antibody could be detected in the gland. More severe lesions were found shortly after the peak anti-thyroglobulin antibody titres. They suggested that anti-thyroglobulin antibody plays an important role in the thyroid lesion.

A correlation between the titre of anti-thyroglobulin antibodies and the degree of cytotoxicity has also been reported in patients with Hashimoto's disease. Calder *et al.* (1973) added normal human lymphocytes and decomplemented sera from patients with Hashimoto thyroiditis to thyroglobulin-coated chicken red blood cells. Significant lysis of red blood cells occurred. This cytotoxicity correlated significantly with the titre of thyroglobulin antibodies in the serum. They proposed that cytotoxic responses could be due to the presence of thyroglobulin antibodies in the form of complexes, either alone or with antigens, on the cells. In addition, they proposed that lymphocytes infiltrating

the thyroid gland could destroy thyroglobulin-coated cells leading to lesions of thyroid tissue.

Further support for a role of antibodies in thyroiditis was provided by Tomazie and Rose (1975) who successfully induced EAT in normal animals by transferring serum from activity immunised mice. Thyroiditis was assessed in their study by lymphocyte infiltration in thyroid glands of recipients. Antibody complexes in the thyroid were thought to be responsible for the thyroid tissue lesion. Nevertheless, they also suggested that sensitised lymphocytes releasing lymphokines into the transferred donor sera could have been responsible for accumulation of macrophages leading to lesions of thyroid tissue.

In spite of the conflicting evidence, it remains reasonable to suggest that humoral responses could play a part, or at least a secondary role, in producing lesions of thyroid tissue.

1.7.4. *Regulation of autoimmune thyroiditis*

The development of autoimmune thyroiditis, both in humans and animals, appears subject to genetic control. In the earlier experiments of Vladutiu and Rose (1971), EAT was induced by inoculating mice with thyroglobulin and FCA. Different strains of mice covering 11 different H-2 haplotypes, showed a correlation between EAT incidence and H-2 haplotypes. Beisel *et al.* (1982) confirmed this interpretation by experiments in which the severity of disease was found to correlate with H-2 haplotypes. Strains susceptible to EAT manifested stronger T lymphocyte proliferative responses *in vitro* (Salamero and Charreire, 1983). The capacity for T cell proliferation could reflect a lack of regulation. Penhale *et al.* (1973 and 1976) showed that autoimmune thyroiditis was induced following neonatal thymectomy and that its severity was reduced after transfer of T lymphocytes. These results have implied that a defect in suppressive influence can lead to autoimmune thyroiditis. Indications of the operation of regulatory mechanisms have also been found in humoral responses. Nakamura and Weigle (1969) were able to transfer EAT into healthy rabbits with sera containing anti-thyroglobulin auto-antibodies only if the sera had been obtained soon

after thyroglobulin challenge of the donor. Serum taken from the donor after full development of thyroid lesions failed to produce damage to recipients. One possibility to explain this could be that a suppressive product was being transferred in the later specimens of serum.

Spontaneously occurring anti-idiotype-antibodies to anti-thyroglobulin autoantibodies have been found in experimental animals and patients with Hashimoto's and Graves' disease. Zanetti *et al.* (1983) detected anti-idiotype-antibodies in Buffalo rats with spontaneous autoimmune thyroiditis. These anti-idiotype-antibodies could react with anti-rat thyroglobulin antibodies and also with lymphocytes from rats with spontaneous or experimentally induced autoimmune thyroiditis. A significant decrease in the level of circulating anti-thyroglobulin antibodies has been found following repeated injection of anti-idiotype-antibodies into Buffalo rats with spontaneous autoimmune thyroiditis. Sikorska (1986) reported the existence of naturally occurring anti-idiotypic antibodies to an anti-human thyroglobulin idiotype in sera of 10% of patients with Hashimoto's and Graves' diseases. Such antibodies might be significant in regulating anti-thyroid autoimmunity.

1.7.5. *The investigation of EAT using cultivated cells*

Investigation of autoimmune thyroiditis using cultivated cells has been undertaken on numerous occasions. It has been utilised either as an accessory technique to *in vivo* studies or as an independent method for *in vitro* research. Cytotoxicity for target cells and proliferation of effector cells have been commonly used as parameters to assess the strength of autoreactivity *in vitro.*

In an earlier study of autoimmune thyroiditis, Pulvertaft *et al.* (1961) added Hashimoto's patients' sera, including complement, to normal human cultured thyroid cells. Damage to cultured thyroid cells was evident from histologic alterations. In contrast, normal morphology was maintained provided the cells were incubated with normal sera. These cytotoxic responses required the presence of complement. In this case, it was unlikely that the cytotoxic response was directed against new antigens

exposed on thyrocytes during enzyme digestion of thyroid tissue, because normal sera did not damage enzyme digested human thyrocytes.

When testing for cell-mediated immune responses, Biorklund (1964) collected thoracic duct lymphocytes from Sprague-Dawley rats immunised with thyroid extract and cultivated them with thyrocytes from normal syngeneic rats. After 24-48 hours of incubation, the thyrocytes were considered to be damaged, on the basis of histologic modification, in 10 out of 17 cases tested. In contrast, Ling *et al.* (1964) failed to observe cytotoxicity for cultured thyrocytes if they were incubated with the lymphocytes from Hashimoto's disease patients. Absence of cytotoxicity was demonstrated by morphological criteria and by the level of isotope release from target cells.

Creemers *et al.* (1983) reported cytotoxic responses against syngeneic thyrocytes after culture with syngeneic lymph node cells from rats immunised with thyroglobulin. Salamero and Charreire (1985) reported that primary sensitisation of spleen lymphocytes by a syngeneic thyroid monolayer could induce cytotoxic responses against isotope labelled target thyrocytes.

In contrast, Kimura and Davies (1991) demonstrated the absence of cytotoxicity to target cells, but the occurrence of lymph node cell proliferation in response to syngeneic thyrocytes. It is possible that the sensitization protocol used in this experiment may have induced only a low level of reactivity, insufficient to produce cytotoxicity.

1.8. Aim of the present investigation

The present study aimed to define mechanisms responsible for negative regulation of autoimmune reactions against thyroid antigens by using cultured cells to monitor the course of an experimental autoimmune thyroiditis animal model. This study was also directed to investigate mechanisms whereby autoimmune responses against peripheral antigens developed. It was necessary, firstly, to adapt culture techniques for thyrocytes from foetal lambs and neonatal rats and to obtain conditions suitable for the occurrence of an autoimmune reaction in culture. Subsequent investigations focussed on

three aspects related to negative regulation mechanisms of EAT. They were the existence of a regulatory mechanism for autoimmunity in normal animals, its influence on pathological autoimmune responses, and the possible cellular mechanisms involved . Finally, attempts were made to determine the extent of participation by peripheral organ-specific antigen in initiation of autoimmune responses and possible mechanisms responsible for peripheral antigen mediated autoimmune response.

The experiments in Chapter 3 deal with the differentiation, propagation and function of foetal lamb's and young rat's thyrocytes in culture. They characterise the optimum circumstances for the maintenance of thyrocytes in culture so that a full range of tissue specific antigens are expressed.

The experiments in Chapter 4 examine the possibility of *in vitro* appearance of autoimmune reactivity against cultivated thyrocytes following *in vivo* interference with development of self-tolerance to thyroid antigens. This test was intended to explore the issue of whether self-tolerance is a basic requirement in order to resist *in vitro* induction of abnormal autoimmune responses. The induction of autoimmune responses was assessed either by cytotoxic responses against target cells or by proliferative responses of effector lymphocytes. Additionally, T subset responsible for the autoimmune responses was examined.

In Chapter 5, the possibility of existence of suppressor factors in normal animals which have experienced the development of tolerance to self-antigens was examined by studying the curtailment of the *in vitro* manifestations of experimental autoimmune thyroiditis which cam be induced by previous interference with self-tolerance to thyroid antigens in foetal life. The lymphocyte subpopulation responsible for suppression of thyroiditis was identified.

In Chapter 6, the strategy of implantation of thyroid allografts during foetal life has been adopted to test the extent of participation of peripheral organ-specific antigen in the initiation of autoimmune responses and possible mechanisms responsible for peripheral autoimmune responses. A series of corresponding *in vitro* experiments using cultivated thyrocytes and lymphocytes was performed. The significance of expression

of class II antigens on cultivated thyrocytes that have been incubated with syngeneic lymphocytes from ^{131}I exposed DA rats was examined.

CHAPTER 2: MATERIALS AND METHODS

2.1. Foetal lambs

Foetal lambs were provided by timed matings of Merino ewes. Surgery was undertaken at 51-54 days gestation (full term is approximately 147 days). All sheep were bred in the Animal Breeding Establishment, John Curtin School of Medical Research .

2.2. Mating of sheep

Adult Merino ewes were prepared for mating by the placement of an intravaginal sponge containing flugestone acetate (40 mg, Chronogest, Laboratoire Pharmacetique, Porges, Paris, France.). Removal of the sponge 10-12 days later was accompanied by an injection of serum gonadotrophin (500 IU, Folligon, Intervet, Australia.). Ewes were then penned with rams and the dates of joining, and of any return joining was recorded, so that the gestation ages of all foetuses were accurately known.

2.3. Identical twin foetal lambs

Identical twin foetal lambs were prepared by surgically splitting blastocysts collected from Merino ewes mated 6 days earlier after induction of multiple ovulation. Each pair of identical twin blastocysts was introduced to the uterus of an unmated ewe in which ovulation had been induced 6 days previously.

2.4. Rats

Inbred DA strain adult rats or 1 week old DA rats were used to provide lymph nodes and spleens or for the preparation of thyrocytes, respectively. All rats were bred in the Animal Breeding Establishment, John Curtin School of Medical Research.

2.5. Mating of rats

One male rat was placed in a cage with a female rat overnight. The time of mating was evaluated by the observation of vaginal plugs. The morning on which a plug was detected was designated as Day 0 of gestation and the day of the birth was designated as Day 0 of the age for young rats.

2.6. Thyroidectomy

To interfere with the development of self-tolerance to the thyroid, foetal lambs' thyroids were removed at 51-54 days of gestation. After starving sheep overnight, surgery was performed. Thiopentone induction was followed by halothane anesthesia. The shaved abdomen was opened via a paramedian incision and the uterus was exteriorised. Uterine muscle and foetal membranes were incised using cautery and the foetal head and neck were delivered. Following a midline cervical incision of the foetal skin and displacement of the sternohyoid muscles, one or both lobes of the thyroid gland were freed from the surrounding tissue by blunt dissection and removed. The incision was closed with 5/0 silk sutures. The foetus and any amniotic fluid that had been collected (with the addition of penicillin) were replaced *in utero* and the membranes and uterine muscle were reconstituted with a series of purse string sutures using size 4 silk. The uterine wound was inverted by means of widely spaced sutures through the serosa to reduce the risk of subsequent adhesions. All the procedures were performed under sterile conditions.

2.7. Inoculation of foetal rats with ^{131}I

Foetal rats of from 17 days gestation each received 200μCi of ^{131}I (New England Nuclear, Boston, MA) in order to interfere with development of the thyroid gland and its organ-specific antigens. Following induction of anaesthesia with ether, the pregnant rat uterus was exposed at laparotomy. Each foetus was inoculated intraperitoneally using stereotaxic apparatus. Following closure of the abdomen, pregnancy usually continued to term.

2.8. Thymectomy

Some foetal lambs were submitted to thymectomy together with thyroidectomy at 51-54 days gestation to examine the influence of thymectomy on the development of anti-thyroid autoimmunity. After anaesthesia of the ewe, uterine muscle and foetal membrane were incised and the foetal head and neck were delivered. A midline incision extending from just rostral to the laryngeal prominence to the xiphisternal notch provided access to the thyroid gland and to the cervical thymus. Following removal of the thyroid gland, cervical and thoracic thymus was removed in one piece by blunt dissection. Wound closure was undertaken as above.

2.9. Thyroid allograft of foetal lambs

Thyroid allografts were implanted at 51-54 days gestation, following thyroidectomy, in some foetuses to test whether autoimmune reactions could be prevented by presentation of organ-specific antigens on non-self cells. An intact lobe of thyroid tissue removed from a foetal donor of similar age was placed in the cavity created by removal of the recipient's gland.

2.10. Syngeneic thyroid grafting of DA rats

Thyroid tissue collected from syngeneic rats after euthanasia with carbon dioxide was implanted under the renal capsule of the recipients. A small incision was made in the renal capsule and, while the edge of this incision was held with forceps, the graft was introduced under the capsule and moved away from the incision. The kidney was then replaced and wound closure was performed.

2.11. The removal of lymph nodes

Prescapsular lymph nodes were removed at *post mortem* from foetal lambs of 100-120 days gestation. The sheep were anaesthetised with pentothal halothane as described above and then, following surgical exposure of the foetus, euthanased by pentothal overdose. In the case of DA rats, cervical, mesenteric and superficial inguinal lymph nodes were removed at 6-12 months of age from these rats after they were killed by carbon dioxide inhalation.

2.12. The removal of the spleen and thymus from DA rats

The spleen and the thymus were removed from some rats so that the effects on a syngeneic thyroid monolayer of cells from these organs could be compared with that of lymphocytes from lymph nodes. Following a midline cervical incision and splitting of the upper part of the sterum, each lobe of the thymus was detached from surrounding tissue by blunt dissection and removed with forceps. The spleen was removed through a left subcostal incision in the abdomen.

2.13. The preparation of thyrocyte suspensions

The preparation of thyrocyte suspensions was performed by the method described by O'Connor *et al.* (1980) with several modifications. Thyroid glands excised from foetal lambs of 51-54 days gestation or from 1 week old DA rats were maintained in Hanks Balanced Salt Solution (HBSS) at 4°C by means of an ice bath around the collection dishes. The glands were freed from connective tissue and diced into 1-2 mm cubes using a surgical blade at 4°C in a laminar air flow cabinet. Enzymatic digestion was carried out by adding a solution containing 0.1%trypsin (Type III, from bovine pancreas, Sigma) and 0.1%collagnase (CLS4, Worthington Biochemical Corporation) in HBSS to the thyroid cubes in a container that was then placed in a shaking waterbath at 37°C. Incubation was performed for 20 min. The supernatant collected was mixed with an equal volume of Modified Eagle's Basal Medium F_{15} (F_{15}) containing 5%foetal calf serum (FCS) and stored on ice. The procedure of digestion of the remaining fragments was then repeated once. The mixture was filtered through a nylon cloth mesh on a fine, stainless-steel wire mesh and centrifuged at 200g at 4°C for 10 min. Finally, the pellet was resuspended in F_{15} containing 5%FCS and stored on ice until use.

2.14.Cryopreservation of thyrocytes from foetal lambs

Freshly isolated thyrocyte suspensions, prepared as described in 2.13, were centrifuged at 200g at 4°C for 10 min. The pelleted cells were resuspended in 5%dimethyl sulfoxide (DMSO, Sigma) in FCS and were immediately transferred to a

freezer at -70°C. After overnight maintenance at -70°C, cells were transferred to a liquid nitrogen freezer at -196°C until required for further use.

When cryopreserved thyrocytes were to be established in culture, the cells were rapidly thawed at 37°C immediately after being removed from the freezer at -196°C. Following admixture with an equal volume of F_{15} (containing 5%FCS), the cell suspension was centrifuged at 200g at 4°C for 5 min. The pellet was resuspended in F_{15} with 5%FCS and was then cultured immediately.

2.15. Thyroid cell culture

The concentration of thyrocytes in the isolated cell suspension was adjusted to 5 $\times 10^6$ cells per ml. The cell suspension was then dropped onto coverslips in culture dishes from a pipette held 2 cm above the coverslip. The dishes were placed in an incubator for 2-3 min before gently adding culture medium taking care not to disturb the cells. The cells were cultured at a density of 0.2-0.5$\times 10^6$ cells per coverslip (13 mm diameter round, Thermanox®, Nunc, Inc. Naperville, Illinois.) which represented a final cell concentration of 0.5-1.5$\times 10^6$ in 3ml of culture medium in a 3.5cm diameter well (6 flat bottom well, 3.5×1.0cm., Linbro®, Flow laboratories, Inc. Virginia, U.S.A.). By carefully dropping the cells onto the culture surface a high local density of cells was achieved, albeit with a lower density of lymphocytes if expressed in terms of total volume of medium. Effectively, 0.2-0.5$\times 10^6$ cells were aggregated within a small area (approximately 0.13cm^2). Cultures were then maintained at 37°C in a gas phase of 95%air and 5%CO_2. The culture medium comprised Modified Eagle's Basal Medium F_{15} containing Penicillin 12mg /100ml, Streptomycin 20mg /100ml, Fungizone 1µg /ml, 5%FCS and Thyrotrophin (TSH) 20mU /ml.

2.16. The preparation of lymphocyte suspensions

Lymphocyte suspensions were prepared from lymph nodes and thymus using the method described by Kleiman *et al.* (1984). Lymph nodes excised from 100-120 day foetal lambs or from adult DA rats were maintained in HBSS solution at 4°C by means of an ice bath around the collection dishes. The nodes were cut into small fragments

with a surgical blade and were then pressed gently through a nylon mesh supported on a fine, stainless-steel wire mesh with the aid of a plastic syringe piston. The single cell suspension was centrifuged at 300g at 4°C for 10 min. The pellet was resuspended in F_{15} medium containing 5%FCS and stored at 4°C until use. Cell counts and dye exclusion were then carried out. All the procedures were performed under sterile conditions at 4°C.

Spleens excised from adult DA rats were diced into small fragments with a surgical blade and pressed through a nylon mesh on a fine, stainless-steel wire mesh with the aid of a plastic syringe piston. A single cell suspension was prepared by centrifugation through Ficoll-paque (Pharmacia) at 300g at 4°C for 15-20min. The intermediate layer was aspirated from the tubes into a new tube, mixed with a equal volume of 5%FCS in F_{15}, and centrifuged at 300g at 4°C for 10 min. The pellet was resuspended in F_{15} containing 5%FCS and stored at 4°C until use.

2.17. Coculture of thyrocytes and lymphocytes

Thyrocytes were incubated at 37°C in a gas phase of 95%air and 5%CO_2 for 3 days (in the case of foetal lamb thyrocytes) or for 5 days (for DA rat thyrocytes). The culture supernatant was discarded before freshly isolated lymphocytes were added to the thyrocyte monolayer at a density of $5\text{-}7\times10^6$ lymphocytes per $0.5\text{-}1\times10^6$ thyrocytes together with 3 ml fresh F_{15} containing 5%FCS and 20mU TSH /ml. The thyrocyte and lymphocyte mixture were maintained at 37°C in the incubator. Replacement of culture medium was carried out by means of a half medium change every 5 days.

2.18. Cytochemical staining

To examine morphological alterations in thyroid monolayers under light microscopy, cells grown on coverslips were fixed with Schaudinn's Fluid and stained with haematoxylin and eosin (H & E).

To monitor the morphology of thyroid monolayers under the electron microscope, cells grown on the coverslips were fixed with 2%glutaraldehyde in 0.1M Na cacodylate buffer at pH 7.4 for 2 hours. Postfixation processing was carried out in

1%osmium tetroxide in the same buffer for 60 min. The cells were stained *en bloc* with 2% uranyl acetate for 60 min after washing with distilled water. Finally, they were dehydrated with a graded series of ethanols and embedded in Spurr's resin.

2.19. Determination of iodide uptake by cultivated thyrocytes

Iodide uptake was measured by the method described by Kraiem *et al.* (1991) with some modifications. Culture medium was removed on the last day of culture and replaced by HBSS containing ^{125}I at a concentration of 2μCi /ml /10^6 cells. This was incubated with the thyrocytes for 60 min at 37°C in a humidified gas phase of 95%air and 5%CO_2. The cells were washed twice with cold HBSS and removed from the surface of the coverslip by enzyme digestion using 0.2%trypsin in HBSS. The cells were then transferred to test tubes and washed twice with cold HBSS. Radioactivity of the pellets resuspended in HBSS was counted in a γ-counter. The total intracellular iodide uptake was expressed as the percentage of radioiodide (^{125}I) bound to cells (RIBC%) that was calculated as shown below. Each count was corrected for non specific background (NSB).

$$RIBC\% = \frac{\text{sample radioactivity (cpm) - NSB (cpm)}}{\text{total iodide radioactivity(cpm) - NSB (cpm)}}$$

Rat epidermal cells were utilised to provide a negative control, because these cells also have an epithelial and fibroblast-like morphology.

2.20. Determination of proliferation of cultivated thyrocytes by measurement of cell numbers and by [H^3]thymidine incorporation into cellular DNA

Growth of cultivated thyrocytes was estimated both by measurement of cell numbers and by [H^3]thymidine incorporation into cellular DNA. Cell numbers were calculated using a haemocytometer after the thyrocytes had been removed from the coverslip by enzymatic digestion with 0.2%trypsin in HBSS.

[H³]Thymidine incorporation into cellular DNA was examined as described in detail previously with minor modifications (Williams 1988). The thyrocytes were cultured at a density of 5×10^4 per well (96 U shaped well, 1.0×0.7cm, Linbro). [H³]Thymidine was added to each well to achieve a final concentration of 2μCi /ml and was incubated with the thyrocytes for 24 hours. After trypsin digestion for 15 min, the cells were collected onto glass fibre filters using an automated cell harvester (1295-004 Betaplate™ 96 well Harvester, Pharmacia). [H³]Thymidine incorporation was measured by means of scintillation counting of dried filters using a 1205 Betaplate™ liquid scintillation counter (Pharmacia). Results were expressed as the mean incorporation of [H³]thymidine. Each sample was examined in triplicate and corrected for non-specific binding. Unlabelled thyrocytes were utilized as the negative control.

2.21. Determination of the proliferation of lymphocytes cultured on thyrocyte monolayers by measurement of [H³]thymidine incorporation into cellular DNA

Thyrocytes were cultured at a density of 5×10^4 per well (96 U shaped well, 1.0× 0.7cm, Linbro) for 5 days. Freshly isolated lymphocytes were added at a density of 5×10^5 per well together with 200μl of fresh F15 containing 5%FCS after discarding the old culture medium. After incubation for 24 hours, [H³]thymidine was added to each well to a final concentration of 2μCi /ml and was incubated with the cells for 24 hours. Following gentle shaking of the culture trays, supernatants containing lymphocytes were aspirated out of each culture well and added to a new well. Cells were then collected onto glass fibre filters using an automated cell harvester. [H³]Thymidine incorporation was measured by scintillation counting of dried filters. Results were expressed as the mean incorporation of [H³]thymidine. Each sample was examined in triplicate and corrected for non-specific binding. Unlabelled lymphocytes were used as a negative control.

2.22. Assessment of severity of damage of thyrocyte monolayers

Damage to thyrocyte monolayers was graded from 0 to 5 on morphologic grounds (Figure 1).

Grade

0. Normal thyrocyte monolayer;
1. Rare foci of slight damage;
2. Infrequent (<5 /coverslip) foci of slight damage;
3. Infrequent (<5 /coverslip) foci of severe damage;
4. Frequent (>5 /coverslip) foci of severe damage;
5. Coalescent foci of severe damage.

Damage was confined to areas in which thyroid follicles had formed. Any areas in which thyrocytes had formed only a non-follicular monolayer remained unaffected.

2.23. Immunocytochemical staining method

To examine the expression of MHC class II antigens on the surface of thyrocyte monolayers, the avidin-biotin immunocytochemical method was used as described by Coggi *et al.* (1986) with several modifications.

Coverslips bearing a thyroid monolayer and lymphocytes were removed from the culture wells. These cells were incubated with rabbit serum (1:50 dilution in phosphate buffered saline) at room temperature (RT) for 20 min. After blotting the excess serum from the coverslips, cells were incubated with mouse anti-rat MHC class II Ia antigen monoclonal antibody (MRC OX-6, Serose) for 60 min at RT and washed 3 times with 0.1%bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 10 min. The cells were then incubated with rabbit anti-mouse IgG antibodies conjugated with biotin at RT for 60 min and washed 3 times with PBS (containing 0.1%BSA) for 10 min. The cells were immediately fixed with 5%acetic acid in 95%ethanol for 10 min and washed 3 times with PBS for 10 min. Quenching of endogenous peroxidase was performed by incubating cells with 0.3%hydrogen peroxide solution in methanol for 20 min before the cells were washed 3 times with PBS. The cells were then incubated with avidin-biotin complex (ABC) reagent (VECTASTAEN® ABC Kit standard, PK-4000, VECTOR laboratories) for 60 min at RT and washed 3 times with PBS. The cells were

subsequently incubated with peroxidase substrate solution which contained an equal volume of 0.02%hydrogen peroxide and 0.1%diaminobenzidine tetrahydrochloride (DAB) in PBS for 7 min. at RT. Finally, these cells were washed 2 times with PBS and then with distilled water for 5 min. Counter staining was carried out with methyl-green pyronin for 1-2 seconds followed by washing with tap water. In each sample, 500 cells were counted to determine the percentage of positive staining.

2.24. Determination of lymphocyte subpopulations by flow cytometry

To observe the percentage of $CD4^+$ and $CD8^+$ subsets in whole populations of lymphocytes from normal or thyroidectomized foetal lambs, the technique of fluorescence activated cell sorting (FACS) has been utilised as described by Foucar *et al.* (1989) with minimal modification.

Isolated lymphocyte suspensions were divided into staining tubes at a density of 10^6 cells per tube. The 10^6 cells were incubated with mouse anti-sheep monoclonal antibodies (undiluted) 100µl for 30 min. at 4°C (on ice). 0.2ml FCS and 1ml HBSS were added to each tube. The cells were mixed gently by pipette and centrifuged at 300g at 4°C for 10 min. After pouring off the supernatant, and blotting the tubes, 100µl of anti-mouse IgG antibody conjugated with fluorochrome per 10^6 cells was added to tubes at a 1:50 dilution and incubated with cells for 15 min at 4°C on ice. This was followed by washing twice with 0.2ml FCS and 1ml HBSS at 4°C. The cells were fixed with 0.5 ml 1%paraformaldehyde, covered with foil and stored at 4°C until being measured by FACScan Flow Cytometer (Becton Dickinson). The monoclonal antibodies included mouse anti-sheep CD4 (SBU-T4) and CD8 (SBU-T8) (Centre for animal biotechnology, The University of Melbourne). Two types of control were utilized including unstained cells and an irrelevant monoclonal antibody (mouse anti-tropomysin monoclonal antibody) labeled cells.

2.25. Separation of lymphocyte subsets by flow cytometry

To identify the lymphocyte subsets responsible for autoimmune reactivity, lymphocytes were sorted by flow cytometry. The procedure was performed as follows:

A suspension of isolated foetal lamb lymphocytes was centrifuged at 300g at 4°C for 10 min.The supernatant was poured off and the excess supernatant was blotted. The cells were then incubated with 50µl /per 10^6 cells mouse anti-sheep CD4 or CD8 subset antibody (undiluted) for 30 min. and agitated gently by pipette for 10 min. After washing once with PBS containing FCS, the cells were incubated with anti-mouse IgG antibodies conjugated with fluorochrome (1:50 dilution in PBS) for 15 min. The cells were then washed twice with PBS and resuspended in F15 at a density of 5×10^6 cells /ml F15. Labelled cell suspensions were sorted by flow cytometer (FACStar plus cell sorter, Becton Dickinson). Labelled cells were excited at 488nm using 200mW laser power. Fluorosceinisothiocyanate (FITC) fluorescent emissions were collected through a 530DF30 filter. Data was collected and analysed using Becton Dickinson lysys II software. After sorting, cells were washed with F15 and resuspended in F15 for culture. All procedures were performed at 4°C under sterile conditions. Two types of control were included using either unstained cells or irrelevant monoclonal antibodies. The recovery rate and viability of cells were determined by measurement of cell numbers and by dye exclusion, respectively. The purity of sorted cell populations was assessed by running small samples of the sorted population through the instrument using the same windows. The purity was then expressed as the percentage of cells falling in the sort gate for the desired cell population exclusive of the percentage of cells falling in the sort gate for the cells that were not required.

2.26. The technique of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of biologically active molecules in culture supernatant

For analysing biological active molecules isolated from culture medium after incubating lymphocytes with thyrocytes, SDS-PAGE was used as described previously by Schagger *et al.* (1987) with several modifications.

2.26.1. *SDS-PAGE solutions*

(All reagents used in SDS-PAGE were produced by BIO-RAD)

(1) *Stock solution of acrylamide and bisacrylamide mixture:* containing 48%acrylamide and 1.5%bisacrylamide. The total monomer concentration (%T) was 49.5%. The crosslinking monomer concentration (%C) was 3%.

(2) *Separating gel solution (with 10%T and 3%C):* containing 2.03 ml the solution described in (1), 3.3 ml gel buffer described in (4), 1.2 ml glycerol, 3.47 ml distilled water, 50µl of 10%ammoniun persulphate solution and 5µl of tetramethylethylenediamine (TEMED).

(3) *Stacking gel solution (with 4%T and 3%C):* containing 0.4 ml solution described in (1), 1.24 ml gel buffer described in (4), 3.36ml distilled water, 25µl of 10%ammonium persulphate and 2.5µl TEMED.

(4) *Gel buffer:* containing 3.0M Tris and 0.3%SDS with a pH 8.45.

(5) *Running buffer:* containing 0.1M Tris, 0.1M Tricine and 0.1%SDS with a pH 8.25.

(6) *Sample buffer:* containing 4%SDS, 12%glycerol, 50mM Tris, 2%mercaptoethanol and 0.001%bromophenol blue.

2.26.2. <u>*Procedure*</u>:

A mini-Protein II dual slab cell was used in this procedure. The cell was sealed first. The separating gel described in 2.26.1. (2) was prepared and added to the cell followed by overlaying with water-saturated isobutanol (upper phase). The gel was set for 30 min until an obvious top could be seen to it . The water-saturated isobutanol was poured off and the gel was rinsed with distilled water. The stacking gel described in 2.261.(3) was then prepared and added into the cell. Combs were placed in position. After setting the gel for 40 min., the stacking gel was washed and filled up with running buffer. After incubating with sample buffer for 30 min. at RT, the samples were added into the gel as 10µl /per well (10 well comb). The samples were run in the stacking gel at 30-40V for 60 min. and at 120-150V in the separating gel for another 60 min. After removing gel from the apparatus, the gels were fixed with 12%trichloroacetic acid (TCA) and stained overnight according to the method of Neuhoff, *et al.* (1988). The staining solution contained 0.1%coomassie brilliant blue R-250 (CBB-R250), 2%phosphoric acid, 6%ammonium sulfate and 20%methanol.

2.27. Protein electroblotting for analysis of biologically active molecules in culture supernatant

For protein sequencing, the proteins separated on SDS gel had to be transferred onto immobilising matrices by protein blotting. The method used was that described previously by Madsudaira (1987) with minimal modifications. The immobilising matrices were prepared from polyvinylidene difluoride (PVDF) non-glass polymer-based membranes. The PVDF membrane, sponges, and filter papers were wetted with 100%methanol for 2-3 seconds and soaked in blotting buffer including 10mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer. The transblot sandwich was assembled in the following order starting from the cathode side: sponges, filter papers, gel, PVDF membrane, filter paper and sponge. The transblot sandwich was then inserted into the cell and electroblotting buffer was poured into the cell. The samples were run at 80-100V at RT for 40 min. After being removed from the transblotting sandwich and rinsed with distilled water, the protein membrane was stained with the solution containing 0.1%CBB-R250, 1%acetic acid and 40%Methanol for 1 min and was then destained twice with 50%methanol. The protein bands of interest were cut off and stored in 4°C for protein sequencing.

2.28. Statistical evaluation

Statistical analyses were by Student's *t* test; *P* values less than 0.05 were considered to be statistically significant.

CHAPTER 3: THE DIFFERENTIATION, MORPHOLOGY, FUNCTION, AND PROPAGATION OF CULTIVATED THYROCYTES FROM FOETAL MERINO LAMBS AND NEONATAL DA RATS

3.1. Introduction

To assess the presence of anti-thyroid autoreactivity in the experiments to be reported in this thesis, cultivated thyrocytes from foetal Merino lambs and neonatal DA rats were utilised as target cells for lymphocyte cytotoxicity. As a pre-requisite, it was necessary first to establish optimum culture conditions for differentiation of thyrocytes from foetal lambs or neonatal DA rats so that the organ specific self-antigens normally expressed by these cells would be retained. The experimental protocol used in the experiments with foetal lambs required the maintenance of foetal lamb thyrocytes *in vitro,* by means of cryopreservation, for periods in excess of 8 weeks. Hence the first requirement was to validate conservation of the antigenicity of cryopreserved thyrocytes.

Cells freshly isolated from thyroid tissue have been used widely in culture to examine thyroid metabolism, the effects of various hormones on thyrocytes, and immune responses against thyrocyte antigens. Techniques using thyrocytes from adult animals have been developed in a number of species including sheep (Kerkof *et al.* 1964a), pig (Lissitzky *et al.* 1971), dog (Winand *et al.* 1975), humans (Bidey *et al.* 1977), and rat (Kerkof *et al* 1966). Both morphology and iodide uptake of cultivated thyrocytes have often been utilised to identify thyrocyte lineages in tissue culture and to observe differentiation or transformation of these cells. Additionally, the course of growth in culture has been used as a means of characterising the differentiation of cultivated cells. Cultivated cells are likely to vary significantly in many properties between their exponential growth and stationary phases. Quantification of growth is also an important element in monitoring a culture in order to select a suitable density of cells.

Pastan (1961), one of the earliest investigators to study isolated adult thyroid cells in culture, used calf thyroid cells isolated by means of enzyme digestion. If the

culture medium did not include TSH, isolated thyrocytes failed to reorganise into the follicles. He found that there was a marked uptake of iodide by thyrocytes after stimulation by TSH.

Soon after Pastan's report, Kerkof *et al.* (1964a) reported that isolated adult sheep thyrocytes in primary culture possessed the ability to reorganise into follicle-like structures if TSH was added to the culture medium. Adrenocorticotropic hormone (ACTH), insulin and plasma albumin were ineffective. He also found that the addition of TSH resulted in an increase in iodide uptake by cultivated thyrocytes. With the aid of electron microscopy, Fayet *et al.* (1971) have demonstrated that, at a suitably high cell concentration and in the presence of TSH, porcine thyroid cells, isolated by trypsinization, aggregated and rearranged into structures possessing characteristics of typical three-dimensional thyroid follicles. These structures resembled cross-sections of intact thyroid tissue. The three-dimensional follicles were characterised by the presence of junctional complexes, differentiated apical poles with microvillae, a follicular lumen and apical vesicles. In contrast, cells cultured in the absence of TSH developed as a two-dimensional layer. No differentiation of their plasma membrane was visible.

In another study of cultivated porcine thyroid cells, Yap *et al.* (1987) found that isolated thyrocytes did not reassociate into functional follicles in the presence of TSH, unless the initial seeding density was adequately high. At a low density (0.2×10^6 cells/3.5cm diameter culture dish), thyrocytes rapidly formed a monolayer in the presence of TSH (128μU /ml). However, delaying the addition of TSH to the culture by 48 hours markedly reduced follicular development, even though cells were seeded at high cell density. They emphasised that the differentiation of thyrocytes in culture required the presence of TSH and that the TSH-responsiveness of thyrocytes in culture was influenced by cell-cell contact.

Most investigators have studied the differentiation of cultivated adult thyrocytes rather than of cells from less mature animals. A few reports have, nevertheless, dealt with the differentiation of cultivated foetal thyrocytes. Huber and Davies (1990) investigated the characteristics of human foetal thyroid cells in culture. They isolated human foetal thyrocytes by means of enzyme digestion and cultured these cells in

medium in the absence of serum. Although colloid droplets were plentiful within thyrocytes, there was no evidence of the formation of follicle or dome structures even when confluent cell growth had occurred. In their report, there was little indication of multiplication of foetal thyroid cells. Although the density of cultivated thyrocytes was not mentioned in this report, it is possible that a slow rate of growth of thyrocytes in serum-free culture medium could have influenced cell-cell contact and so affected the differentiation of these cells.

In most studies of thyrocyte cultivation, follicle structure has been reported to remain intact for a limited period of 7 to 10 days. Thereafter, follicles have usually disappeared rapidly and the culture has developed as a conventional monolayer (O'Connor *et al.* 1980). Provided the medium was not changed in primary cultures, follicular morphology could usually be maintained for more than 20 days (O'Connor *et al.* 1980). Regular changes into fresh medium caused cultures to lose their differentiated characteristics and to revert to a conventional monolayer. In the experiments of O'Connor *et al.,* the capacity to trap iodide could be preserved for at least 20 days, provided TSH was present and medium changes were avoided. Under these culture conditions, cells underwent little proliferation.

Alternatively, by using a cloning technique, Ambesi-Impiombato *et al.* (1980) were able to preserved thyrocytes for 3 years in a differentiated form in medium with a low concentration of serum. However, the success of cloning techniques when applied to animal cell culture has been limited by the poor cloning efficiency of most primary cultures (Freshney, 1990b). Another problem is that animal cells may only survive for a limited number of generations. Consequently, they might already be close to senescence by the time a clone was produced. In addition, contamination with bacteria occurred readily during long term culture (Freshney, 1990b).

The studies in this chapter will focus on examination of the differentiation, morphology, function, and propagation of isolated thyrocytes from foetal lambs and neonatal DA rats in culture. I will also examine the influence of cell density, serum concentration, TSH concentration, and different programs of medium change on the differentiation, morphology, propagation, and function of cultivated thyrocytes. An

account will be given of a cryopreservation technique which was utilised to preserve the specific properties of foetal lamb thyrocytes *in vitro*. Following this, the cultivation characteristics of frozen thyrocytes from foetal lambs will also be examined.

3.2. Characteristics of differentiation, morphology, function and proliferation of cultivated thyrocytes from foetal lambs and neonatal DA rats in primary culture

Both frozen, thawed thyrocytes from foetal lambs of 51-54 day gestation and freshly isolated thyrocytes from neonatal DA rats have been observed consistently to reorganise into follicle-like or dome structures in culture medium containing 5%FCS and 20mU /ml TSH using the culture method described in Chapter 2.15 (Figure 2). These follicle-like structures were formed between 24 and 48 hours after initiation of culture and were completely developed after 3 to 4 days incubation. Under electron microscope examination, these structures appeared as typical three-dimensional follicles with microvillae and follicular lumina (Figure 3). Numerous microvilli were visible at the apex of the cells. These structures could usually be maintained for 15-20 days, depending on the protocol for medium changing and on the concentration of serum in the culture medium.

In a culture medium containing 5%FCS and 20mU /ml TSH, frozen thyrocytes from foetal lambs or freshly isolated thyrocytes from neonatal DA rats exhibited mean values (±SE) of 0.36%±0.04% or 0.28%±0.03% respectively of radioiodine (^{125}I) bound to cells (RIBC) during the first 15 days of incubation. Thyrocytes had a significantly higher capacity ($P<0.01$) for ^{125}I uptake than did DA rat epidermal cells that possessed a mean value (±SE) of 0.06%±0.01% of RIBC (Figure 4). The iodide uptake peaked after 2 days of incubation and progressively decreased after 15 days of incubation.

Both frozen foetal lamb thyrocytes and freshly isolated neonatal DA rat thyrocytes had a doubling time of 4 hours and a short log phase of 2 days. The growth of freshly isolated rat thyrocytes then entered the plateau phase in contrast with cryopreserved foetal lamb thyrocytes which continued to proliferate moderately after 2 days of incubation (Figure 5-6).

3.3. The influence of cell density on differentiation, morphology, function and proliferation of thyrocytes

Cell density has been considered to influence directly the formation of follicles (Yap *et al.* 1986). The influence of various cell densities has been examined by using the culture methods described by Yap *et al.* (1986). Increasing cell numbers up to 4×10^6 in the 3.5cm diameter well (about 0.05×10^6 cells /per $0.13cm^2$) did not enhance the formation of follicular structures (Figure 7). Follicular structure only developed in regions where numerous cells had been deposited. It could be readily attained over most of the coverslip by using the method described in Chapter 2.15. In this, more than 0.2×10^6 thyrocytes were dropped on and accumulated in a small area of about $0.13cm^2$. The cell density per $0.13cm^2$ in the present experiments was about 4 times that used in Yap's experiment. Nevertheless, the total cell concentration ($0.5\text{-}1.5\times10^6$ cells in 3ml culture medium on a $9.6cm^2$ area (3.5cm diameter well)) was lower than that of $3\text{-}4\times10^6$ cells in the same volume described by Yap *et al.* (Table 1). Under the light microscope, overlap of follicles could be clearly observed in the present experiment (Figure 7).

There was no significant difference, as indicated by either iodide uptake or proliferation of thyrocytes, between the method described by Yap and the method used in the present experiment at the same cell concentration.

3.4. A comparison of morphology, function and proliferation of freshly isolated and cryopreserved thyrocytes in culture

After undergoing cryopreservation, foetal lamb thyrocytes still possessed the properties of freshly isolated cells including consistent formation of follicular structures (Figure 2 and 3) and iodide uptake (Figure 4). In the case of foetal lambs, there was no significant difference between the iodide uptake of freshly isolated thyrocytes with a mean value (±SE) of RIBC of 0.30%±0.03% and cryopreserved thawed thyrocytes with a mean value (±SE) of 0.36%±0.04% (Figure 8). Both cryopreserved and freshly isolated foetal lamb thyrocytes had a doubling time of 4 hours. After 2 days log phase, freshly isolated thyrocytes no longer proliferated whereas cryopreserved cells continue to proliferate moderately (Figure 9-10).

3.5. Influence of concentration of TSH in culture medium on the differentiation, morphology, function, and proliferation of thyrocytes

Six concentration levels of TSH have been examined, namely 0, 10, 20, 40, 80, and 100mU/ml. When using the culture method described in 2.15. and a culture medium supplemented with serum, a follicular structure developed, regardless of the presence of TSH in the culture medium. However, only a few follicles appeared in the absence of TSH in contrast to the numerous follicles formed in its presence (Figure 11). Nonetheless, high concentrations of TSH (more than 30mU /ml) in culture medium did not further enhance the formation of follicles. Isolated foetal lamb or neonatal DA rat thyrocytes did not reassociate into a follicular structure in the absence of both TSH and serum.

Compared with cells maintained in the absence of TSH in the culture medium (RIBC=0.1%), thyrocytes in the culture medium containing TSH (10mU /ml) manifested a considerably higher value of 1.2%RIBC when iodide uptake was tested after 2 days of initiating culture of the thyrocytes. There was, however, no progressive increase in the value of RIBC accompanying increase in the concentration of TSH once this exceeded 20 mU /ml (Figure 12).

Figure 13 shows the influence of TSH concentration in the culture medium on the proliferation of thyrocytes when [H^3]thymidine incorporation into rat thyrocytes was determined 24 hours after initiating culture of thyrocytes. The [H^3]thymidine incorporation increased along with increase in the concentration of TSH. A TSH concentration of 80mU/ml in the culture medium produced the maximum proliferation of cells.

3.6. The influence of serum concentration in the culture medium on thyrocyte differentiation, morphology, function, and proliferation

Five levels of serum concentration were examined in this experiment (10%, 5%, 2.5%, 0.5% and 0%). Follicular structure could develop in culture medium without serum, or with any concentration of serum, if the culture method mentioned in 2.15. in

the presence of TSH was used. Follicles appeared at about 24 hours of incubation in culture medium with 5-10%FCS and after about 3-5 days in culture medium with 2.5%FCS. These structures could be maintained for 5-7 days in culture medium with 10%FCS, and for 10-15 days in culture medium containing 5%FCS. However, cells in culture medium with a low serum concentration (<2.5%) grew so slowly that only a few follicles appeared. On the other hand, with high concentrations of serum (10%) in the culture medium, fibroblast-like cells grew so fast that they covered the follicles (Figure 14). Follicles could be produced and maintained satisfactorily in culture provided cells were incubated in culture medium with 5%FCS for 3-5 days and then kept in the culture medium with 2.5%FCS thereafter.

Within 15 days of initiating cultivation, thyrocytes in medium with low serum concentrations showed a slightly lower capacity for iodide uptake (mean values ±SE of 0.23%±0.03%RIBC) than did cells in medium with 5%FCS which had a mean value (± SE) of 0.28%±0.03%RIBC. Additionally, decline in iodide uptake by thyrocytes in culture medium with 0.5%FCS started at the eleventh day of incubation. This was earlier than the time (the fifteenth day of incubation) at which this was observed for thyrocytes in culture medium with 5%FCS (Figure 15).

Thyrocytes in culture medium with either 5%FCS or 0.5%FCS showed an increase in [H^3]thymidine incorporation within 24 hours of incubation. However, there was little increase in cell numbers at any time and decrease in numbers occurred earlier (8 days) in the case of thyrocytes in culture medium with 0.5%FCS (Figure 16-17). In culture medium containing 5%FCS, thyrocyte numbers increased markedly within 2 days of cultivation and then the proliferation of thyrocytes entered the stationary phase. Thyrocyte numbers in this medium were decreasing after 14 days of cultivation (Figure 16-17).

3.7. The influence of various protocols for medium change on thyrocyte differentiation, morphology, function, and proliferation

Medium changes, at either 2-3 days or 5-7 days, induced overgrowth of fibroblast-like cells covering the follicles. Follicles could be maintained satisfactorily in

culture provided that the medium was changed to a low concentration of serum after the first 5 days of incubation. This was achieved by retaining half of the primary medium and replacing only half with fresh medium. Failure to change the medium resulted in the death of the thyrocytes. Follicular structures were lost if subculture was performed at any stage of incubation. Different protocols of medium change did not induce obvious differences in cell proliferation or in iodide uptake.

3.8. Discussion

The experimental results in this Chapter have defined the characteristics of cryopreserved foetal lamb and freshly isolated neonatal DA rat thyrocytes in culture. These cultivated cells have consistently reorganised into follicular-like or dome structures in culture medium with 5%FCS and TSH 20mU /ml using the culture method described in Chapter 2.15. Under the electron microscope, these structures appeared as typical three-dimensional thyroid follicles characterised by the presence of junction complexes, differentiated apical poles with microvillae and follicular lumina. These cells were effective at iodide uptake by comparison with epidermal cells. This function was well maintained for 15 days.

When cultured under similar conditions, thyrocytes from both sources have begun to proliferate within 2 days of commencing incubation. Thereafter, neonatal DA rat thyrocytes entered a plateau phase while frozen foetal lamb thyrocytes still exhibited moderate proliferation until the 15th day of incubation. These results suggested that, after isolation by enzyme digestion, cryopreserved foetal lamb thyrocytes and neonatal DA rat thyrocytes retained their specific properties under suitable culture conditions.

If a conventional culture method in which the cells were spread onto the culture surface was employed, the increase of cell density was inadequate for formation of follicle structure. In contrast, these structures were readily formed using the method described in Chapter 2.15. in which a high density of cells ($0.2\text{-}0.5\times10^6$ cells /$0.13cm^2$ area) was placed onto a small area of the culture surface. Under the light microscope, closely opposed follicles could be observed. This suggested that organisation of thyroid follicular structure required both a high density of cells and the aggregation of these

cells in a three-dimensional structure as in the organ of origin. Both increasing culture seeding density and creating conditions favouring cell clustering could enhance cell-cell contact and so promote intercellular cooperation and communication (Yap *et al.* 1987). Facilitation of gap junction communication could assist with the operation of second messengers such as cyclic AMP (Finbow and Pitts, 1981).

High cell density could influence the differentiation, growth and capacity of thyrocytes for iodide uptake. Maintenance of these cell properties usually requires a decrease in the cell numbers by subculture (Freshney, 1990b). However, the culture method described in Chapter 2.15 achieved high cell densities in only a small region whereas cell concentration in the total culture volume remained low. In this culture situation, thyrocytes maintained their capacity for iodide uptake well and commenced proliferation within 2 days of incubation. As the starting number of thyrocytes obtainable from foetal lambs was low (a problem compounded by 40% loss of cells after cryopreservation), a culture method which facilitates proliferation is an advantage.

In these experiments, TSH played an important role in the formation of follicle structure, iodide uptake and cell proliferation. In the absence of TSH and serum, isolated thyrocytes did not reassociate into follicular structures, even though maintained under conditions of high cell density. The manner in which TSH influenced the reorganisation of follicles has been documented. Lissitzky *et al.* (1971) showed that TSH stimulates a 2 to 3-fold increase of cyclic AMP concentration in cultured isolated thyroid cells within 10 minutes. They showed that the action of TSH could be explained by the activation of adenyl cyclase so that cyclic AMP synthesis was increased. This led to the synthesis of a specific RNA. Reorganisation of thyrocytes into follicles required new RNA synthesis which in turn directed the formation of proteins involved in intercellular recognition, aggregation and organisation of thyroid cells.

The presence of TSH in the culture medium effected a marked increase in the function of iodide uptake by cultivated thyrocytes in addition to enhancing the formation of thyroid follicles. This correlation is in accord with the expectation that iodine metabolism in the thyroid is dependent on intact follicular structure (Lissitzky *et al.* 1971). Lissitzky *et al.* showed that iodide uptake did not occur if thyrocytes formed

simple monolayers, without follicles. TSH addition was ineffective in stimulating follicle formation and iodide metabolism if it was deferred for longer than 24 hours after initiating a culture. This also suggested the importance of follicle organisation in the maintenance of specialised thyrocyte functions.

Formation and maintenance of follicular structure were directly influenced by the concentration of serum. High concentrations of serum (10%) induced rapid formation of follicles. It is possible that serum contained growth-stimulating factors (Sato, 1975). These factors could stimulate the activation of adenyl cyclase leading to an increase of cyclic AMP synthesis. However, high concentrations of serum in culture medium also induced overgrowth of fibroblast-like cells at the expense of follicles. It is likely that the suitability of low concentrations of serum for follicle maintenance reflects their retarding effect on growth (Ambesi-Impiombato *et al.* 1980).

Low concentrations of serum also restricted the formation of follicles and iodide uptake. Consequently, it was necessary to develop a protocol which used different serum concentrations for establishment and maintenance of follicles.

The various programs of medium changes which were tested did not reveal obvious differences as regards iodide uptake and cell proliferation. However, frequent changes of culture medium induced overgrowth of fibroblast-like cells. Follicle structure could be well maintained provided the medium was changed to achieve a low concentration of serum after the first 5 days of incubation, or was changed by retaining half of the primary medium and replacing only half with fresh medium. A possible explanation for this requirement is that a low concentration of serum in the culture medium could limit growth of fibroblast-like cells, and might thereby preserve follicle structures. A half medium change might also achieve a low serum concentration because thyrocytes could take up serum from the primary culture medium.

CHAPTER 4: MANIFESTATIONS OF AUTOIMMUNE REACTIONS AGAINST CULTIVATED THYROCYTES

4.1. Introduction

As described previously in Chapter 1.5.5., Eishi and McCullagh (1988) and McCullagh (1989) have described two animal models in DA rats and foetal Merino lambs in which autoimmune thyroiditis has been induced *in vivo* by interference with thyroid self-recognition during foetal life. Autoimmunity was demonstrated in each species by challenge with syngeneic or self thyroid in later life. To explore the mechanisms of pathogenesis and regulation of the autoimmune responses operative in these models in more detail, it is necessary to develop techniques for their study *in vitro*. The first requirement, consequently, is to establish reproducible assays for monitoring autoimmune responses against cultivated thyrocytes.

Autoimmune thyroiditis in experimental animals and in humans involves responses mediated either by autoantibodies produced by B cells or by antigen-specific T lymphocytes. Although the issue of which type of response is of greater importance remains controversial, the more common interpretation is that cell-mediated autoimmune responses play the primary role in the induction of autoimmune thyroiditis (described in Chapter 1.6.4. and 1.6.5.). In this thesis, I intend to examine mainly cell-mediated responses in autoimmune thyroiditis.

Both cytotoxicity responses against target cells and proliferative responses by effector cells have been commonly used to analyse cell-mediated components of autoimmune thyroiditis *in vitro*. Either morphological modification of, or isotope release from, target cells has been utilised to identify the occurrence of cytotoxic responses. Induction of proliferative responses by effector cells has usually been examined by measuring [H^3]thymidine incorporation into cellular DNA.

The feasibility of induction of cell-mediated autoimmune thyroiditis *in vitro* remains uncertain and has been long debated. Biorklund (1964) placed thoracic duct lymphocytes from Sprague-Dawley rats, immunized with thyroid extract and Freund's complete adjuvant, onto cultivated thyrocytes from normal syngeneic rats. In 10 out of

the 17 cases tested, cytotoxic responses were considered to have occurred as judged by morphologic modification of the cultivated thyrocytes after 24-48 hours of incubation. In the positive cases, thyroid epithelial cells displayed degeneration and necrosis. In the most extreme cases, numerous thyrocytes were lysed and only a few cells remained. Clumping or agglutination of the lymphoid cells alone was interpreted as the expression of a mild response. In response to the observation that some cases exhibited only moderate destruction of cultivated thyrocytes, Biorklund suggested that this might reflect the use of a whole thyroid extract rather than of soluble antigens, when immunization was performed *in vivo.* Another possibility, namely that the lymphocyte donors may have been killed before the height of their immune response was reached, was considered.

In contrast, Ling *et al.* (1965) were unable to detect cytotoxic responses against cultivated human thyrocytes after incubating them for 48 hours with lymphocytes from patients with Hashimoto's disease. Similar results were found, in the case of cultivated rat and guinea pig thyrocytes, after incubation with lymphocytes from syngeneic rats or guinea pigs immunised with whole human thyroid extracts. In no instance did they succeed in demonstrating degenerative structural changes in thyroid cells, nor was it possible to show a specifically increased release of isotope from these cells, after incubating them with the corresponding lymphocytes. Under comparable conditions, however, sera from these individuals were cytotoxic.

The proliferative response of effector cells has also been widely utilised to identify the occurrence of autoimmune responses. Charreire's group (Yeni and Charreire, 1981; Salamero and Charreire, 1985) established an EAT model *in vitro.* In this model, cultivated thyrocytes from CBA mice were incubated with syngeneic spleen lymphocytes that had been sensitised previously by exposure to syngeneic cultivated thyrocytes. When compared with lymphoid cells that had been cultivated alone, spleen lymphocytes previously incubated with thyrocytes manifested a significantly high level of [H^3]thymidine incorporation on re-exposure to thyrocytes. However, lymph node or thymus cells from the same mice, which had also had previous exposure to thyrocytes, were only slightly stimulated. When thyroid-sensitised spleen lymphocytes were

deposited on ^{51}Cr labelled thyrocytes as target cells, syngeneic cytotoxicity responses occurred after 5 hours of incubation. These experiments revealed a correspondence between the cytotoxic response against target cells and proliferative responses of effector cells.

Kimura and Davies (1991) examined the induction of autoimmune thyroiditis *in vitro* in cells from Wistar rats by following both proliferative responses of effector lymphocytes and cytotoxic responses against target thyrocytes. Both effector lymphocytes and target thyrocytes were derived from normal syngeneic rats. Whereas they were unable to detect cytotoxic responses, as indicated by isotope release from target thyrocytes, [H^3]thymidine incorporation by lymphocytes increased markedly in comparison with that by cultivated lymphocytes alone. In these experiments, the effector lymphocytes had not been previously sensitised by exposure either to thyroglobulin or to thyrocytes, other than to the extent that this can occur in normal rats. It is unlikely that effector lymphocyte proliferative responses provide a more accurate indication of immunisation, because normal lymphocytes that had not been previously stimulated by thyroid antigens also mounted proliferative responses. In contrast, the induction of cytotoxic responses was restricted by the form of immunisation, the type of antigen and the strain of animals.

T cells are an important factor in the induction of autoimmune responses. The identity of the lymphocytes responsible for induction of autoimmune thyroiditis is a basic issue requiring further investigation and is not universally agreed. Examination of the subpopulation of lymphocytes infiltrating thyroid tissue of patients with Hashimoto's disease by Canonica *et al.* (1985) revealed that a $CD8^+$ subset was the main population. These $CD8^+$ cells proliferated when they were confronted by human thyroglobulin antigens. In contrast, these is a higher percentage of B cells than T cells within the thyroid of Hashimoto's thyroiditis patients. This accords with the high rate of local synthesis of thyroid-directed antibodies that was observed (Jansson *et al.* 1983).

In this Chapter, I will describe the establishment of an *in vitro* experimental autoimmune thyroiditis model based on the *in vivo* animal models described by Eishi and McCullagh (1988) and McCullagh (1989). In the case of foetal lambs, the thyroids

were removed at 51-54 days of gestation. Excised thyroids were then converted into single cell suspensions by enzyme digestion and stored at a temperature of -196°C for 60-70 days. At 100-120 days of gestation, lymph nodes were obtained from these thyroidectomized foetal lambs. Freshly isolated lymphocytes were then cultured with autologous thyrocytes that had been thawed and cultured for 3 days previously (Figure 18).

In the case of rats, foetal DA rats of 17 days gestation received a dose of ^{131}I sufficient to disrupt further development of the thyroid gland. This was administered shortly after the capacity to concentrate iodide was acquired by the foetal thyroid. This could be expected to interfere with the expression of thyroid specific antigens that normally accompanies tissue differentiation. This effectively prevented the development of self-tolerance of those antigens. One year later, lymph nodes were removed from the treated rats, which were then adult, and cell suspensions were prepared. Freshly isolated lymphocytes were incubated with syngeneic thyrocytes that had been cultured previously for 5 days (Figure 19).

After coculture for 5 or 10 days, the severity of any cytotoxic responses was measured by observation of any morphological modifications of the cultivated thyrocytes. Effector lymphocyte proliferative responses were also monitored by measuring [H^3]thymidine incorporation at 4-48 hours of incubation. The influence on the outcome of these two types of assay of variation in a number of parameters including cell numbers, culture time, and challenge of the animal's immune system by previous implantation of syngeneic rat thyroid tissue under the renal capsule have been examined. As serum has been proposed as a factor that could stimulate immune responses (Yeni and Charreire, 1981), the influence of serum on the proliferative responses of lymphocytes and on their cytotoxic responses against thyrocytes were also studied. The induction of autoimmune responses was also compared in hemi-thyroidectomized and completely thyroidectomized foetal lambs in order to verify that the autoimmune responses observed in the latter were directed against non-transformed thyroid antigens. This was undertaken to exclude the possibility that enzyme digestion and cryopreservation had produced modified antigens which were the target of any

observed cytotoxicity. The effects of lymphocytes from different rat organs on the induction of autoimmune responses were examined.

Additionally, T lymphocyte subsets isolated from foetal lambs by flow cytometry were incubated with autologous thyrocytes in order to identify the T subset responsible for autoimmune responses.

4.2. The occurrence of autoimmune reactions against cultivated thyrocytes

4.2.1. *Cytotoxic responses against foetal lamb thyrocytes*

After 10 days incubation with lymphocytes from a thyroidectomized foetal lamb, cultivated autologous thyrocytes had undergone severe degeneration with necrosis in all 10 cases tested (mean grade 4.7±0.2). Major destruction was apparent at the apices of epithelial thyrocytes which had organised into thyroid follicles. These cells showed cloudly swelling, hyaline-like degeneration and lysis. The interconnection of follicles was almost completely lost, being replaced by single, deformed, degenerated "follicles" or areas of coverslip with no follicles. In severe cases, complete lysis of thyrocytes has been observed (Figure 1). In contrast, most areas of cultivated autologous thyrocyte monolayers that had been incubated with lymphocytes from the normal identical foetal co-twin of the thyrocyte donor or with lymphocytes from hemi-thyroidectomized foetal lambs retained intact thyroid follicles with their distinctive structure of follicle epithelial cells (mean grade 1 or 1.2±1.0 respectively). Slight damage, including cell atrophy and detachment from the coverslip surface, were occasionally observed in some foci (Table 2. and Figure 1.). Similar features have been observed in thyrocyte monolayers from the same foetuses that had been incubated without lymphocytes. The mean grade of damage to thyrocyte monolayers after incubation with lymphocytes from thyroidectomized foetal lambs was significantly higher ($P<0.01$) than the mean grade in hemi-thyroidectomized or normal co-twin foetuses.

Nine foetuses, which had been submitted to bilateral thyroidectomy at 51-54 days of gestation, were found to possess residual thyroid tissue at *post mortem* at 100-

120 days gestation. After incubation with autologous lymphocytes for 10 days, cultivated thyrocytes in 7 out of these 9 cases retained intact follicle structure, but 2 cases developed severe degeneration with necrosis of thyrocytes (Table 3).

4.2.2. *Cytotoxic responses against DA rat thyrocytes*

In the case of 17 out of the 23 (74%) ^{131}I exposed DA rats which were tested, cultivated normal syngeneic thyrocytes were damaged after incubation with their lymphocytes. The thyrocytes in 10 of these 17 cases showed foci of moderate damage. In the other 7 cases, severe damage and cell lysis was observed (Table 4 and Figure 20). Thyrocytes that were incubated with normal syngeneic lymphocytes, or in the absence of lymphocytes, retained normal follicular structures with foci of slight destruction observed only occasionally.

Apart from damage to thyrocytes, lymphocyte clumping over follicles was observed in most instances of cells from thyroidectomized or ^{131}I exposed individuals, but this phenomenon was also seen in several normal cases. Its significance remains unclear. There was no apparent penetration of lymphocytes into thyrocytes that could be observed under the light microscope.

4.2.3. *The proliferative responses of lymphocytes, on exposure to thyrocytes, as assessed by [H^3] thymidine incorporation assay*

To ascertain the time of maximum proliferation, measurement of [H^3]thymidine incorporation into lymphocytes has been performed after 3 different times of incubation namely 4, 24 and 48 hours. After 24 hours of incubation with syngeneic thyrocytes, lymphocytes from both normal and ^{131}I exposed rats manifested their highest mean values (of 1902±57.2 CPM and 1979±221.1 CPM respectively) for [H^3] thymidine incorporation (Table 5).

The mean values of 1902±57.2 CPM and 1979±221.1 CPM respectively of [H^3] thymidine incorporation by lymphocytes from normal and ^{131}I exposed rats after 24 hours of incubation with syngeneic thyrocytes, compared with mean values in lymphocytes cultivated alone for 24 hours of 199±28.6 CPM and 272±68.2 CPM

respectively (The increase produced by exposure to thyrocytes was statistically significant, $P<0.01$). There was no significant difference between the mean values of [H^3]thymidine incorporation by lymphocytes from normal and ^{131}I exposed rats after incubation with syngeneic thyrocytes (Table 6. and Figure 21). (Note: In the case of coculture of lymphocytes and thyrocytes, the mean value of [H^3]thymidine incorporation was obtained only from the lymphocytes in the supernatant from the coculture. As described in Chapter 2.21., before the extent of [H^3]thymidine incorporation by lymphocytes cultured with thyrocytes was measured, the supernatant from the mixed culture was aspirated and added to a new well after incubation with thyrocytes for 24 hours and [H^3]thymidine for 24 hours. The mean value has also been corrected for non-specific binding, including the mean value of [H^3]thymidine incorporation of supernatant from thyrocytes cultured alone.)

A similar outcome was observed when the normal and thyroidectomized co-twins of 2 pairs of identical twin foetal lambs were compared. Lymphocytes from the normal or thyroidectomized co-twin, cultivated alone, had mean values (±SE) of 781±161.4 CPM and 825±43.4 CPM of [H^3]thymidine incorporation, respectively. After incubation with autologous thyrocytes, lymphocytes from both types of foetus showed higher mean levels of incorporation 2527±214.3 CPM and 2563±78.8 CPM. However, once again, there was no significant difference between the mean values observed with lymphocytes from the two sources (Table 7).

4.3. The influence of culture environment on the manifestation of in vitro EAT

4.3.1. *The influence of lymphocyte numbers on manifestation of cytotoxic responses*

Six different aliquots (2, 5, 7 ,10, 12 and 15×10^6) of autologous lymphocytes from thyroidectomized foetal lambs were separately cultured with 10^6 thyrocytes. Table 8 shows the results. Severe destruction was not observed in cultivated thyroid monolayers incubated with 2×10^6 lymphocytes. Three out of 5 cases (60%) showed severe destruction of thyrocytes when incubated with 5×10^6 lymphocytes. After incubation with 7-12×10^6 lymphocytes, severe destruction of thyrocytes was seen in all

instances. On exposure to 15×10^6 lymphocytes, thyrocyte monolayers degenerated with numerous thyrocytes detaching from the monolayer in 3 out of 5 (60%) cases. This phenomenon could also be observed on the thyrocyte monolayer after incubation with 15×10^6 normal lymphocytes. However, thyrocytes incubated with 15×10^6 normal lymphocytes retained intact cell membranes, even though the monolayer became atrophic and detached from the coverslip. This contrasted sharply with thyrocyte monolayers exposed to autologous lymphocytes from thyroidectomized foetuses which sustained destruction of the follicles with severe degeneration and lysis (Figure 1).

In the case of ^{131}I exposed rats which were not subjected to challenge with syngeneic thyroid implants *in vivo,* lymphocytes from 10 out of 23 cases tested induced only moderate damage of thyrocyte monolayers, even though 10^7 lymphocytes were used. However, a second batch of thyrocyte monolayers were severely damaged by culture with 5×10^6 lymphocytes in all 10 of these cases after they had been challenged by syngeneic thyroid *in vivo.* Severe atrophy and detachment of the thyrocyte monolayer could also be observed after incubation with 15×10^6 lymphocytes from normal syngeneic DA rats.

4.3.2. *The influence of culture time on manifestation of cytotoxic responses*

Six thyroidectomized foetal lambs were used to provide lymphocytes for examination of the influence of culture time on autologous thyrocyte damage. Four different culture times were tested namely 2, 5, 7 and 10 days. After incubation with lymphocytes from thyroidectomized foetal lambs, there was no destruction of thyrocytes after 2 days of incubation. Thyrocytes had sustained a moderate degree of destruction in 3 out of 6 cases, while, in the remaining 3 cases, most follicle structures remained intact after 5 days of culture. In all cases, damage to thyrocytes was apparent by 7 days, although in 2 cases this was only moderate. Severe destruction of thyrocytes was observed in all cases after 10 days incubation (Table 9).

4.3.3. *The influence of serum in culture medium on the development of cytotoxic or proliferative responses*

The thyrocytes from the normal and thyroidectomized co-twins of 2 pairs of identical twin foetal lambs of 51 days gestation were cultured with their lymphocytes at 110-120 days gestation. Culture was undertaken either in medium containing 5%FCS or in serum free medium in order to examine the influence of serum on the development of cytotoxic and proliferative responses.

When serum free culture medium was utilized, severe atrophy has been observed on the part of thyrocytes from either the normal or thyroidectomized co-twin of all foetal lambs at 5 days of incubation. Numerous thyrocytes detached from the monolayer, passing into suspension at 10 days of cultivation.

4.4. Cytotoxic responses of lymphocytes from hemi-thyroidectomized and incompletely thyroidectomized foetal lambs

Seven foetal lambs were submitted to hemi-thyroidectomy. The follicular structure of autologous thyrocytes monolayers remained substantially intact after 10 days of incubation with autologous lymphocytes from all of these lambs. Severe damage to thyrocytes was not observed in any of these cases (Table 2). In another 9 foetal lambs, the intention had been to perform bilateral thyroidectomy, but some residual thyroid tissue was found at *post mortem.* In 7 of these cases, normal thyroid follicle structure was preserved by monolayers incubated with autologous lymphocytes, whilst in the 2 remaining cases severe destruction of thyrocytes occurred (Table 3). In these 2 cases, the volume of residual thyroid was found to be less than 3×3mm.

4.5. The influence of challenge of ^{131}I exposed rats with syngeneic thyroid implants on the subsequent cytotoxicity of their lymphocytes for thyrocyte monolayers

The cytotoxic capacity, for syngeneic thyrocyte monolayers, of lymphocytes from rats exposed to ^{131}I in foetal life was compared before and after challenge of the donor with a syngeneic thyroid graft. It has been demonstrated that syngeneic thyroid tissue, implanted in adult life under the renal capsule of ^{131}I exposed rats is subject to autoimmune thyroiditis (Eishi and McCullagh, 1988).

In 10 cases, moderate destruction of thyrocytes was observed after incubation with lymphocytes from ^{131}I exposed rats which had yet to receive thyroid implants. However, a second batch of thyrocyte monolayers were severely damaged by culture with lymphocytes from the same group of rats *after* they had been challenged by syngeneic thyroid *in vivo*.

In another 6 ^{131}I exposed rats, normal thyrocyte structures were generally retained and only slight destruction occurred in some areas of syngeneic monolayers occurred after incubation with their lymphocytes. Nevertheless, 2 out of these 6 cases showed severe destruction of thyroid monolayers cultured with their lymphocytes from the same ^{131}I exposed rats *after* challenge with syngeneic thyroid *in vivo*. (Table 10).

4.6. Comparison of the cytotoxic capacity of lymphocytes from different lymphoid organs of ^{131}I exposed rats

Extensive lysis of thyrocyte monolayers (grade 5) was observed at an earlier time (at 5 days of incubation) when spleen cells, instead of lymph node cells from DA rats exposed to ^{131}I in foetal life were incubated with them. The degree of thyrocyte destruction after incubation with lymph node cells was also lower (grade 2 at 5 days of incubation) than that achieved by spleen cells from the same rats. In contrast, thyrocytes incubated with thymus cells from these rats remained undamaged. Thyrocytes were also unaffected by incubation with thymus or lymph node cells from normal syngeneic DA rats. However, in all of 8 normal DA rats tested, severe thyrocyte destruction (grade 5) also occurred after 5 days of incubation with their spleen lymphocytes (Table 11).

4.7. Identification of the lymphoid cell subset responsible for induction of autoimmune thyroiditis against cultivated thyrocytes

Four different FACS-separated lymphocyte subpopulations from thyroidectomized foetal lambs were prepared in order to test their reactivity against autologous thyrocytes. They were CD4 selected, CD4 depleted, CD8 selected, and CD8 depleted subpopulations. It has been reported that a monoclonal antibody directed against CD4 interfered with the function of CD4 cells (Nabozny *et al.* 1991). For this

reason, CD4 depleted or CD8 depleted subsets, which included CD8 or CD4 cells respectively, together with other cell types, were utilised as a basis for comparison with positively selected subpopulations. This was considered to provide a test for possible interference with the function of cells targeted by monoclonal antibodies.

4.7.1. *Dilution of antibodies*

To examine the phenotype of the T subset responsible for the induction of autoimmune responses against cultivated thyrocytes, indirect antibody techniques have been used. In this technique, cells were first stained with a non-fluorescent primary monoclonal antibody which was then detected by the binding of a secondary antibody conjugated to a fluorochrome. Appropriate dilutions of antibodies have been determined by antibody titration against a fixed number (10^6) of cells. The point at which saturation of binding sites occurred was defined on the flow cytometer when increasing antibody concentration produced little increase in fluorescence intensity.

A fixed number of 10^6 lymphocytes from the normal co-twin of a thyroidectomized identical co-twin foetal lamb were incubated with 100µl of primary antibody, either anti-CD4 or anti-CD8, undiluted or in dilutions of 1:1, 1:5 or 1:10. The cells stained with undiluted anti-CD4 or anti-CD8 antibody displayed the highest intensity of positive fluorescence (Figure 22). This indicated that undiluted primary antibodies of both anti-CD4 and anti-CD8 were at a saturating dilution.

At a fixed dilution (undiluted) of primary antibody (either anti-CD4 or anti-CD8), 10^6 lymphocytes from the normal identical co-twin foetal lamb were incubated with 100µl of secondary antibody (rabbit anti-mouse IgG antibody conjugated with fluoresceinisothiocyanate (FITC)) in the following dilutions: undiluted, 1:25, 1:50, 1:100 and 1:200. Cells stained with this secondary antibody at a 1:25 dilution manifested the highest intensity of positive fluorescence (Figure 23). This indicated that a 1:25 dilution of secondary antibody was a saturating dilution. The 1:50 dilution of this secondary antibody used in this group of experiments was not a saturating dilution.

4.7..2. *Percentage of CD4$^+$ and CD8$^+$ subsets in whole populations of lymph node cells from thyroidectomized foetal lambs*

Table 12 shows the percentage of CD4$^+$ and CD8$^+$ lymphocyte subsets among whole populations of lymph node cells from thyroidectomized foetal lambs as determined by flow cytometer. There was a mean value (±SE) of 31.5%±3.3% for the CD4$^+$ subset and a mean of 16.9%±1.5% for the CD8$^+$ subset. To test the relative reactivity of different subpopulations, 10^6 thyrocytes were incubated with 3-4×10^6 autologous CD4-selected cells, 7×10^6 CD4-depleted cells, 1.5-2×10^6 CD8-selected cells or 8×10^6 CD8-depleted cells, respectively.

4.7.3. *Recovery rate, death rate and purity of lymphocytes after sorting by flow cytometer*

After sorting by flow cytometer, 70% of the total sorted lymphocytes were recovered, of which 96% were viable. The purity of lymphocyte subsets was in excess of 97%.

4.7.4. *Specific phenotype of autoreactive cells*

Table 13 shows the results of these tests with lymphocyte subpopulations. Most thyrocyte monolayers retained a normal structure after incubation with autologous CD4-selected lymphocytes. A similar outcome was observed after incubation of autologous thyrocytes with CD8-selected cells in all 5 cases tested or with CD8-depleted populations in all 4 cases tested. However, severe destruction of thyrocytes was observed after incubation with a CD4-depleted population in all 6 tested cases.

4.8. Discussion

Following 10 days incubation with autologous lymphocytes from thyroidectomised foetal lambs of 100-120 days gestation, cultivated thyrocytes from all 14 foetal lambs tested were subject to severe damage. Damage was usually confined to thyrocytes which had organised into follicles. These cells displayed cloudy swelling, hyaline-like degeneration and lysis leading to the loss of an interconnected follicular

structure and its replacement by single, deformed, degenerate "follicles" or by bare areas. Complete lysis of thyrocytes was observed in severe cases. Nevertheless, thyrocytes cultured with lymphocytes from the normal identical twins of thyroidectomized foetuses, or from hemi-thyroidectomized foetal lambs, retained completely connected follicle structures with clear, intact membranes at the apices of the cells forming the follicles. These experimental results showed that *in vitro* cytotoxic responses could be induced following the introduction of autologous lymphocytes onto cultivated thyrocytes from individual foetuses that had failed to develop thyroid self tolerance.

In the case of 17 out of the 23 ^{131}I exposed DA rats tested (74%), cultivated syngeneic thyrocytes were damaged after incubation with their lymph node lymphocytes. Thyrocytes incubated with lymph node lymphocytes from normal, syngeneic DA rats retained an intact follicle structure. These results indicated that *in vitro* cytotoxic responses occurred following introduction of lymphocytes from ^{131}I exposed DA rats onto cultivated syngeneic thyrocytes. Nevertheless, in comparison with foetal Merino lambs in which severe cytotoxic responses were observed in all 14 cases tested, severe cytotoxic responses were manifested by lymphocytes from only 7 out of 23 (30%) ^{131}I exposed DA rats tested. Ten out of 23 (43%) cases displayed moderate damage and 6 out of 23 (26%) cases retained their normal follicle structure. This difference may have reflected differences in the protocols employed to destroy thyroid tissue in lambs and rats.

Lymph node lymphocytes from the 10 out of 17 ^{131}I exposed DA rats that exhibited positive cytotoxic responses, produced only moderate destruction of thyrocytes. Additionally, lymphocytes from 6 of the ^{131}I exposed rats failed to show cytotoxic responses. However, after incubation of a second batch of monolayers with lymphocytes from ^{131}I exposed rats that had been stimulated by implantation of thyroid tissue, all 10 cases which had been scored as moderate cytotoxic responses previously and 2 out of 6 cases previously unresponsive were observed to be subject to more severe damage. One possible explanation for this could be that autoreactive lymphocytes had clonally expanded after stimulation by thyroid antigens *in vivo*. This

could have augmented the cytotoxic impact of these lymphocytes when they encountered the same antigens *in vitro*. Whereas cytotoxicity towards syngeneic thyrocyte monolayers was confined to lymph node lymphocytes from ^{131}I exposed rats, splenic lymphocytes proved to be cytotoxic irrespective of whether they had been harvested from normal or ^{131}I exposed rats.

In both sheep and rat experiments, thyroid cells for cultivation were prepared by means of enzyme digestion. It has been suggested that this could modify the structure or function of cells (Edwards and Fogh, 1959). Edwards and Fogh observed morphologic changes in human amnion cells that had been exposed for 1 hour to 0.25 per cent trypsin. The modifications were most marked at the cell border, where the basement membrane was completely lacking and the microvilli absent. They suggested that the digesting effect of trypsin on cell membranes, results in the unmasking of antigenic determinants. In the present experiments, thyroid cells were prepared by means of enzyme digestion. However, destruction of such enzyme-exposed thyrocytes was not observed when they were cultured either with normal lymphocytes or in the absence of lymphocytes. It is unlikely that the observed cytotoxic responses were initiated by the exposure of a new group of antigens induced by enzyme digestion. One enzyme, collagenase, has been considered to make thyroid cells less sensitive to cytotoxicity (Biorklund, 1964). This could have the effect of protecting thyrocytes.

The possibility should be considered that cryopreservation of foetal thyrocytes might facilitate exposure of new antigenic groups on the cells and so predispose them to evoke immune responses. This appears not to have occurred as thyrocytes from hemi-thyroidectomized or bilaterally thyroidectomized foetuses in which residual thyroid inadvertently remained, or thyrocytes which were exposed to lymphocytes from normal identical co-twins of thyroidectomized donors had also been maintained by cryopreservation, and yet were not subject to cytotoxic responses when exposed to autologous lymphocytes. Consequently, it is likely that adequate cryopreservation did not alter the original antigenic characteristics of thyrocytes.

In the present experiments, the induction of cytotoxic responses was obviously influenced by the numbers of cultivated lymphocytes from foetal lambs. When cultured

with 10^6 thyrocytes from thyroidectomized foetal lambs, 2×10^6 autologous lymphocytes failed to produce damage. In 60% of cases, thyrocyte destruction occurred after incubation with 5×10^6 autologous lymphocytes. Marked damage occurred among thyrocytes incubated with $7\text{-}12\times10^6$ autologous lymphocytes. The relationship observed between thyrocyte destruction and lymphocyte numbers could be due to a requirement for an augmenting effect of lymphokines secreted by reactivated lymphocytes to achieve cell-mediated immune responses. It may be necessary for lymphokine concentration to attain a certain level that constitutes a threshold for reactivity. Low numbers of lymphocyte producing only limited quantities of lymphokines would then fail to produce effects.

Excess numbers of lymphocytes, irrespective of their origin from normal or autoimmune foetuses, resulted in detachment of thyrocyte monolayers from their coverslips. This effect was produced with 15×10^6 lymphocytes. Despite detachment of the monlayers, any specific damage produced by the lymphocytes could still be observed. Thus thyrocytes incubated with 15×10^6 normal lymphocytes retained intact cell membranes, even though detached from the coverslips. In contrast, thyrocyte monolayers exposed to 15×10^6 autoreactive lymphocytes were characterised by the features of damage previously described (Figure 1). Monolayer detachment could be due to deficient nutrition in culture medium after uptake of nutrients by excess lymphocytes.

The cytotoxic responses observed during the present experiments resembled delayed type immune responses *in vivo* to the extent that severe destruction only occurred after 7 days of incubation. This suggests that failure to observe cytotoxic responses in some of the reports reviewed in the introduction of this Chapter could have been attributable to restriction of observation to a short time (usually within 48 hours) of incubation. The interval elapsing between introduction of lymphocytes to the thyrocyte monolayer and the development of cytotoxicity could also reflect a requirement for proliferation of an initially small number of reactive cells.

Long term primary culture itself might result in cell degeneration. The experimental results that were described in Chapter 3, nevertheless, showed that

thyrocytes were able to maintain their specific characteristics *in vitro* for at least 15 days. The capacity to take up iodide decreased after this time. Significantly, intact follicle structure was also retained after thyrocytes were cultured with *normal* lymphocytes. This strongly suggests that the cytotoxic responses observed when thyrocytes were placed in contact with autologous lymphocytes from a *thyroidectomized* foetus were not induced by degeneration of thyrocytes as a result of prolonged maintenance in culture.

Serum has been considered to stimulate immune responses (Yeni and Charreire, 1981). Yeni and Charreire (1981) tested the influence of sera from different species on lymphocyte proliferative responses against thyrocytes. Lymphocyte proliferative responses were expressed by [H^3]thymidine incorporation into cellular DNA. They showed clear-cut inhibition of [H^3]thymidine incorporation when syngeneic or allogeneic sera were incorporated in the culture medium. In contrast, a marked enhancement occurred when a xenogeneic serum (FCS) was incorporated in the culture medium. In the present experiments, the use of serum-free culture medium retarded thyrocyte growth. Cells detached from the coverslip under these conditions and re-entered the supernatant, an occurrence which could be mistaken, on superficial examination for cytotoxicity. On the other hand, in culture medium with 5%FCS, sheep or rat thyrocytes cultured with lymphocytes from normal, autologous foetal lambs or normal syngeneic DA rats, respectively, retained an intact follicle structure. This indicated that the addition of adequate serum to the culture medium does not produce false positive cytotoxic responses.

As described in Chapter 1.5.5., thyroid hormones have been shown to affect the development and function of the immune system. Removal of the thyroid gland, or interference with its the development, could result in the occurrence of autoimmune thyroiditis. In the present experiments, none of the foetal lambs which had been hemi-thyroidectomized became autoimmune as assessed by the development of cytotoxic responses against autologous thyrocytes on the part of their lymphocytes. In addition, 5 out of 7 cases in which presumed bilateral thyroid removal was disproved at *post mortem* by the finding of residual thyroid tissue, failed to manifest cytotoxic responses.

It is highly unlikely that the cytotoxic responses observed in present experiments were influenced by the deficiency of thyroid hormone. In the 2 other cases, cytotoxicity to cultivated thyrocytes was observed following their incubation with autologous lymphocytes. In these 2 cases, the volume of residual thyroid was much smaller, being less than 3mm×3mm. The likely explaination for development of autoimmunity in these animals is that self-tolerance development may require the availability of relevant self-antigens above a certain level.

The capacity of lymphocytes from different lymphoid organs to mount autoreactive cytotoxic responses *in vitro* has also been examined. The cytotoxic response against rat thyrocytes observed when they were incubated with lymph node cells from ^{131}I exposed rats was specific in that lymph node cells from normal rats failed to induce cytotoxic responses. However, spleen lymphocytes from either normal or ^{131}I exposed rats induced an earlier occurrence of more intensive cytotoxic responses than did lymph node or thymus cells. Thyrocytes retained their normal structure after incubation with thymus cells from either normal or^{131}I exposed rats. These results indicate that the cytotoxic response of lymph node cells from ^{131}I exposed rats against syngeneic thyrocytes is specific. The reactivity of spleen cells from normal rats against syngeneic thyrocytes may be explicable in terms of the numerous red cells that remained after passage of spleen cells through ficol-paque. Incubation of lymphocytes with syngeneic red cells has been reported to induce cytotoxic responses against syngeneic red cells (Charreire *et al.* 1974). This may conceivably have increased their cytotoxic response against thyrocytes. On the other hand, cytotoxicity of normal spleen cells might indicate the of existence of anti-thyroid autoreactivity in <u>*normal*</u> rats. The existence of autoreactive lymphocytes in normal individuals has been reported by several investigators. Pereira *et al.* (1985) showed that, in normal non-immunised mice, $L3T4^+$ cells could induce proliferation and antibody secretion by normal B lymphocytes. Lyt-2^+ cells efficiently suppressed B lymphocyte responses. Guilbert *et al.* (1982) reported the existence of natural antibodies against 9 common antigens (tubulin, actin, thyroglobulin, myoglobulin, fetuin, transferrin, albumin, cytochrome c and collagen) in the sera of normal individuals. In the present experiments, the activities

of lymphocytes might be expected normally to be controlled *in vivo* by suppressor cells. Under the altered conditions prevailing *in vitro*, however, it is possible that these cells might escape from suppressor influences leading to cytotoxic responses. Additionally, cytotoxicity on the part of normal lymphocytes was detected only in spleen cells in the present experiments. One possible explanation for this could be that suppressor cells might be confined predominantly to other lymphoid organs.

In the present experiments, lymphocyte proliferative responses have also been examined. After incubation with autologous or syngeneic thyrocytes for 24 hours, lymphocytes from thyroidectomized foetal lambs or from ^{131}I exposed rats, respectively, exhibited high mean values of [H^3]thymidine incorporation as compared with lymphocytes cultured alone. However, lymphocytes from normal individuals of both species also showed the same high mean values after incubation with thyrocytes. This suggested that a lymphocyte proliferative response could be a normal response, not correlated with cytotoxic activity. Positive proliferative responses of effector lymphocytes in association with negative cytotoxic responses towards target thyrocytes have been reported also in reactions between cultivated thyrocytes and *normal* syngeneic lymphocytes (Kimura *et al.* 1991a, b). In those experiments, the lymphocytes had not been deliberately stimulated previously by any thyroid antigens. This suggested that, in studying autoimmune responses, lymphocyte proliferative responses were not useful in identifing the induction of pathological autoimmune responses.

The experimental results reported in Chapter 4.7 indicated that a CD4 depleted population of lymph node cells from thyroidectomized foetal lambs was competent to mount cytotoxic responses against autologous cultivated thyrocytes. This active population could have included a $CD8^+CD4^-$ subset. The presence of reactivity in CD4 depleted populations implied that $CD8^+$ cells were responsible for the induction of the anti-thyroid autoimmune responses. This finding was not surprising given that a $CD8^+$ population has been identified as the major contributor to the T lymphocytes infiltrating affected thyroid tissue from patients with Hashimoto's disease (Canonica *et al.* 1985). Canonica *et al.* proposed that this $CD8^+$ population included cytotoxic cells. In the

present experiments, the requirement for $CD8^+$ cells for autoimmune cytotoxicity is in accord with their proposal.

The experimental result in Chapter 4.7 also revealed that a $CD8^+$ selected population of lymph node cells from thyroidectomized foetal lambs was not cytotoxic for an autologous thyroid monolayer. One possibility to explain this negative outcome is that dendritic cells, macrophages or B cells, which exist in a $CD4^+$ depleted population but not in a $CD8^+$ selected population, participate in the process of activation of $CD8^+$ cells. Schuler *et al.* (1985) found that although sensitised T lymphocytes were stimulated by cells bearing the relevant MHC gene products, freshly isolated Langerhans' cells were comparatively inefficient stimulators of T lymphocyte activation *in vitro*. However, dendritic cells from draining nodes were more efficient in activation of T lymphocytes (Cumberbatch *et al.* 1991). It has been found that an intimate association of lymphocytes with dendritic cells preceded lymphocyte transformation and cytokine synthesis (Inaba *et al.* 1983, Flechner *et al.* 1988).

CHAPTER 5: THE INVESTIGATION OF REGULATION MECHANISMS FOR EXPERIMENTAL AUTOIMMUNE THYROIDITIS

5.1. Introduction

The clonal deletion theory originally predicted that normal individuals would lack autoreactive cells, because these cells had been eliminated during the development of self-tolerance. According to this theory, autoimmune responses were regarded as completely pathological phenomena induced by re-emergence of autoreactive immunocomponent cells as a consequence of exposure to cross reactive antigens or to the modification or mimicking of self-antigens. Inhibition of immune system function would then be the only way in which to manipulate autoimune responses.

In the light of "immune suppression" theories, suppression effects have been proposed as a means of regulating the development of self tolerance. These effects could take the form of negative regulation of the function of the autoreactive cells that have been demonstrated to persist in normal individuals, with the result that self-tolerance was established and maintained. Pathological autoimmune responses would then be envisaged, not as the consequence of re-emergence of autoreactive cells, but as resulting from defects in the suppression process. A logical consequence of an immune suppression theory would be to place emphasis on investigation of regulation of autoimmune responses in normal individuals as a means of providing insight into the effective regulation of pathological autoimmune responses.

The fundamental question which must be addressed in deciding whether suppression of autoimmune reactivity is central to maintaining self tolerance, rather than being an epiphenomenon, is whether suppression can be demonstrated in *normal* individuals. The existence of suppressor mechanisms has been demonstrated in some animal models of artificially induced immunological tolerance. McCullagh (1970) transferred thoracic duct lymphocytes from normal syngeneic donors to rats tolerant to sheep erythrocytes and found that the transferred lymphocytes failed to induce haemolytic antibody responses in the recipients. Gershon and Kondo (1971) transferred spleen cells from mice made tolerant to sheep red blood cells to normal syngeneic mice.

They observed the prevention of immune responses to sheep red blood cells in the normal recipients. In their experiments, immune suppression was shown to be mediated by thymus-derived, antigen-specific lymphocytes. The existence of suppressor cells related to overt autoimmune responses has been reported. Penhale *et al.* (1973, 1976) found that neonatal thymectomy could induce thyroiditis. The transfer of normal syngeneic thymus cells into thymectomized animals inhibited the induction of this autoimmune thyroiditis. McCullagh (1990) parabiosed normal DA rats with syngeneic rats which had previously been submitted to interference with the development of self-tolerance to thyroid antigens by inoculation with ^{131}I *in utero*. Subsequently, syngeneic thyroid lobes were introduced under the renal capsule of both parabionts. He found that no instance of thyroiditis was observed in implants in either the ^{131}I exposed or the normal partner, provided parabiosis had been undertaken before, or simultaneously with, graft implantation. In contrast, thyroiditis was evident in implanted thyroid grafts in ^{131}I exposed rats alone or in both partners' implants if the normal parabiont had received irradiation before surgery. These results suggest that normal DA rats possess migratory, radiosensitive cells with the capacity to curtail any expression of anti-thyroid reactivity by rats in which thyroid development has been disrupted before the development of immunocompetence.

The phenotypic identity of suppressor cells has long been debated. Chen *et al.* (1992) demonstrated the generation of antigen-specific suppressor T cells following the experimental induction of tolerance to ovalbumin (OVA) in mice. These suppressor T cells were clonally maintained *in vitro* and periodically stimulated with OVA. The feeder cells were able to suppress *in vitro* antibody production in an OVA-specific and MHC class I restricted manner. All of these T suppressor cell clones were showed to be Thy 1.2^+, $CD4^-$, $CD8^+$ and to express CD3 and the $\alpha\beta$ heterodimer of the T cell receptor.

It has been suggested that some $CD4^+$ cells may have a suppressive capacity. Thomas *et al.* (1981) found that, although pokeweed mitogen-activated $OKT4^+$ cells could function as helper cells *in vitro*, these activated $OKT4^+$ cells could also inhibit B

cell differentiation induced by fresh OKT4$^+$ cells, This suppressor function was radiosensitive.

Unique surface markers for T suppressor cells have yet be detected, and this has led to questioning of the existence of suppressor effects (Moller, 1988). Nevertheless, several investigators have proposed that suppression could require a number of cell interactions. It was envisaged that different types of T cells might play a role in suppression effects. After finding two additional antigens, namely the 2H4 and 4B4 molecules, expressed on T lymphocytes, Morimoto *et al.* (1985) proposed that CD4$^+$ 4B4$^+$ cells acted as helper-inducer cells, while CD4$^+$2H4$^+$ cells were suppressor-inducer cells. This suggested that CD4$^+$ suppressor cells might play an inducer role during the development of suppression.

Alternatively, it is conceivable that conventional helper and cytotoxic T cells might, under certain conditions, function as T suppressor cells, or some T suppressor cells might be conventional T lymphocytes with partial or modified function. Pereira *et al.* (1988) proposed that the ability of cytotoxic T lymphocytes to mediate either cytolytic function or non-cytolytic suppressor function was dependent on their stage of maturation or activation.

Many antigens, including thyroid specific antigens, are considered to be expressed only on non-lymphoid peripheral tissues (Lo, 1992). Hence, this type of antigen could not be transported to the thymus to be recognised by thymus cells (Posselt *et al.* 1992). Consequently, doubts exist about the role of thymus in regulation of autoimmune responses initiated by peripheral antigens. However, in the experimental system of Penhale *et al.* (1973 and 1976), neonatal thymectomy predisposed to development of autoimmune thyroiditis in rats whereas transfer of thymus-derived cells from syngeneic rats prevented the response, suggesting an important role for the thymus in the regulation of autoimmune responses initiated by peripheral antigens.

In this Chapter, I first examined *in vitro* the possibility of a suppressor effect in normal individuals that naturally tolerate self-antigens. It is likely that, if a suppressor mechanism is operative in normal self-tolerant individuals, it should be capable of regulating autoimmune responses in a genetically identical individual after loss of self

tolerance. To test this hypothesis, identical twin foetal lambs were utilised. One of each pair of identical twin foetal lambs of 51-54 days gestation was submitted to bilateral thyroidectomy. The thyroid glands were then converted to cell suspensions and stored at a temperature of -196°C for more than 8 weeks. As described in Chapter 4, thyroid ablation at 51-54 days interfered with the subsequent development of tolerance of thyroid-specific self antigens. This was reflected in the occurrence of autoimmune responses against cultivated thyrocytes when these cells were incubated with autologous lymph node cells from the same foetus collected after it had attained 100-120 day gestation. Lymphocytes from the normal co-twin would not induce autoimmune responses against the identical twin's thyrocytes, because this foetus has success fully passed the stage of establishment of tolerance to thyroid self-antigens. If lymphocytes from normal and thyroidectomized foetuses encounter each other, as indicated above, the suppressor cells among the normal co-twin's lymphocytes would be expected to inhibit autoimmune responses against thyroid self-antigens that would otherwise be initiated by the lymphocytes of the thyroidectomized co-twin. (Figure 24).

In normal DA rats, the existence of suppressor cells was also examined *in vitro* by examining the possible regulation of autoimmune thyroiditis in ^{131}I exposed rats (described in Chapter 4) by lymphocytes from normal syngeneic rats that were naturally tolerant to thyroid self-antigens. Thyroid glands from normal DA rats were converted into single cell suspensions that were then cultured for 5 days. Lymph nodes from normal and ^{131}I exposed DA rats were removed and were separated into single cell suspensions. These cells were then incubated with cultured thyrocytes from normal syngeneic rats. (Figure 25).

The specific phenotypic identity of the cells responsible for suppression was examined by introducing isolated T lymphocyte subsets from normal co-twin foetal lambs and whole populations of lymphocytes from thyroidectomized co-twin foetuses onto monolayers of thyrocytes from the thyroidectomized co-twin foetal lamb. Isolation of normal lymophocyte subpopulations was performed by flow cytometry. As antibodies might have the capacity to interfere with the function of lymphocytes to

which they bind (O'Rourke and Mescher, 1993), the reactivity of CD4 and CD8 depleted populations was tested in addition to that of $CD4^+$ and $CD8^+$ populations.

One group of foetal lambs were submitted to both thyroidectomy and thymectomy in order to explore the effect of the thymus on regulation of autoimmune responses against thyroid antigens.

5.2. The regulation of autoimmune thyroiditis by lymphocytes from normal, self tolerant animals

5.2.1. *Negative regulation, by lymphocytes from a normal co-twin, of pathological autoimmune responses mounted in vitro by lymphocytes from its thyroidectomized identical co-twin*

Ten pairs of identical twin foetal lambs were examined in this experiment. There was no more than slight destruction (mean grade 1.3±0.2) of thyrocytes after their incubation with lymphocytes from the normal foetal co-twin in any of these 10 cases. Slight destruction, manifested by cell atrophy and detachment of thyrocytes from the culture surface, was occasionally observed in some foci, but most thyrocytes remained in intact thyroid follicles with their distinctive structure of follicle epithelial cells. Severe destruction (mean grade 4.5±0.2) of thyrocytes occurred following their incubation with lymphocytes from the thyroidectomized foetal co-twin in all 10 tested cases. This severe destruction was accompanied by cloudly swelling, hyaline-like degeneration and lysis at the apices of thyrocytes organised into follicles. Incubation with a mixture of lymphocytes from both co-twins resulted in curtailment (mean grade 2.5±0.2) of the destruction otherwise observed following exposure to lymphocytes from the thyroidectomized co-twin alone in 9 out of 10 cases (Table 14 and Figure 26). In these cases, most follicle epithelial cells retained their distinctive structure. Foci of degeneration of thyroid follicles were occasionally found. The mean grade (2.5) of damage to thyrocyte monolayers after incubation with a mixture of lymphocytes from both co-twins was significantly decreased ($P<0.01$) in comparison with the mean grade

(4.5) of damage to thyrocyte monolayers incubated with lymphocytes from the thyroidectomized co-twins

5.2.2. *Negative regulation, by lymphocytes from normal DA rats, of pathological autoimmune responses mounted in vitro by lymphocytes from ^{131}I exposed rats*

The results presented in Chapter 4.2.2. and 4.5. showed that lymphocytes from 10 out of 23 ^{131}I exposed rats produced only moderate damage in cultivated thyrocytes whereas lymphocytes from all of these rats produced severe damage in thyroid monolayers when the donor had been challenged with a syngeneic thyroid implant. As it would be difficult to assess the extent of any suppressor cell-mediated curtailment of cytotoxic responses if these were only of moderate intensity, the ^{131}I exposed rats used as lymphocyte donors in this experiment were all previously challenged *in vivo* with syngeneic thyroid implants.

Twelve ^{131}I exposed and 12 normal syngeneic rats have been examined. After incubation with lymphocytes from ^{131}I exposed rats which had received renal subcapsular implants of syngeneic thyroid, thyrocytes sustained considerable damage (mean grade 4.3±0.1). In contrast, most thyrocytes retained their normal structure after incubation with lymphocytes from normal syngeneic rats (mean grade 1.1±0.1). After incubation with a mixture of lymphocytes from normal and ^{131}I exposed rats, thyrocytes were protected from severe damage in 9 out of 12 (75%) cases tested (mean grade 2.6± 0.3) (Table 15 and Figure 27). The mean grade (2.6) of damage to thyrocyte monolayers after incubation with a mixture of lymphocytes from normal and ^{131}I exposed rats was significantly decreased ($P<0.01$) in comparison with the mean grade (4.3) of damage to thyrocyte monolayers after incubation with lymphocytes from the ^{131}I exposed rats.

5.2.3. *Influence of normal cell numbers on curtailment of cytotoxicity*

As 5×10^6 lymphocytes from thyroidectomized foetal lambs regularly produced severe damage in autologous thyrocyte monolayers, this number was selected for testing the suppressor capacity of lymph node cells from normal identical co-twins. In this

group of experiments, lymphocytes from the normal foetal lamb co-twin or from normal DA rats were added to the thyrocyte monolayers 2 hours earlier than the lymphocytes from the thyroidectomized co-twin or ^{131}I exposed rats.

Different numbers of lymphocytes from the normal foetal co-twins were selected for incubation with lymphocytes from thyroidectomized foetuses. These included 5, 7, and 10×10^6 cells. Four pairs of identical twin foetal lambs were examined. The addition of 5×10^6 lymphocytes from normal foetal lambs, failed to avert severe damage to thyrocytes in 2 of the 4 cases. However, curtailment of destruction occurred in all cases tested with 7×10^6 normal lymphocytes. (Table 16).

In DA rats, addition of 5×10^6 normal lymphocytes failed to curtail the autoimmune response in 3 out of 8 tested cases. When 7×10^6 normal lymphocytes were used, the curtailment of the autoimmune responses occurred in all 4 cases tested (Table 15).

5.2.4. *Influence on curtailment of cytotoxicity of the interval between placement of normal lymphocytes on thyrocyte monolayers and the addition of lymphocytes from animals autoreactive against thyroid antigens*

In the case of identical twin foetal lambs, curtailment of the destruction of autologous thyrocytes occurred in all 8 cases in which lymphocytes from the normal co-twin were placed on the thyrocyte monolayer 2 hours earlier than lymphocytes from the thyroidectomized foetus. If lymphocytes from both sources were simultaneously added to the monolayer, curtailment of the damage was not observed in 2 out of 5 cases (Table 17).

A similar finding in relation to timing of introduction of normal and autoreactive cells onto thyrocyte monolayers has been found using DA rats. When normal lymphocytes and lymphocytes from ^{131}I exposed rats were simultaneously added, damage to thyrocytes was not reduced in 5 out of 6 cases. However, if normal lymphocytes were placed on thyrocyte monolayers 2 hours earlier than lymphocytes from an ^{131}I exposed rat, damage to thyrocytes was reduced in all cases tested (Table 18).

5.3. Examination of the phenotype of T suppressor cells

5.3.1. *Percentage of CD4$^+$ and CD8$^+$ subsets in whole populations of lymph node cells from normal identical co-twins*

Preparatory to selecting numbers of CD4$^+$ and CD8$^+$ lymphocyte from the normal co-twin appropriate for co-cultivation with whole populations of lymphocytes from thyroidectomized co-twins, the percentage of the CD4$^+$ and CD8$^+$ subsets in whole populations of lymph node cells from normal co-twins has been examined by flow cytometry. Table 19 showed that there were means of 32.4±6% of CD4$^+$ subset cells and 15.7±1% of CD8$^+$ subset cells in the 5 foetuses examined.

5.3.2. *Recovery rate, death rate and purity of lymphocytes after sorting by flow cytometer*

As indicated in Table 20, after sorting by flow cytometer, 70% of the total sorted lymphocytes were recovered, of which 96% were viable.

Table 21 and Figure 28-29 demonstrate the purity of lymphocyte subsets achieved after sorting by flow cytometry. The purity of sorted cell populations was assessed by running small samples of the sorted population through the instrument using the same windows. The purity was then expressed as the percentage of cells falling in the sort gate for the desired cell population exclusive of the percentage of cells falling in the sort gate for cells that were not required.

5.3.3. *Specific phenotype of suppressor cells*

After lymphocyte sorting, four categories of lymph node cell subpopulations were obtained including CD4$^+$, CD4 depleted, CD8$^+$ and CD8 depleted populations. Taking account of the percentages of CD4$^+$ and CD8$^+$ subsets in whole lymphocyte populations as indicated above, 3-4×10^6 CD4$^+$, 6-7×10^6 CD4 depleted, 1.5-2×10^6 CD8$^+$ and 8×10^6 CD8 depleted populations from normal co-twins were co-cultivated

with 5×10^6 whole population lymphocytes from the corresponding thyroidectomized co-twin.

When the $CD8^+$ lymphocyte subpopulations from normal co-twin foetal lambs were mixed with whole populations of lymphocytes from the corresponding thyroidectomized co-twins, thyrocytes were protected from damage. In contrast, the 3 remaining lymphocyte subpopulations from normal co-twin foetal lambs failed to curtail the destruction of thyrocytes (Table 22).

5.4. Influence of thymectomy on the occurrence of autoimmune responses.

A group of foetal lambs was submitted to surgical removal of both thyroid and thymus glands at 51-54 days gestation. In these cases, severe damage to thyrocyte monolayers appeared earlier than was observed in the case of cells from foetuses which had been thyroidectomized only. After 5 days of incubation with lymphocytes from thyroidectomized-thymectomized foetuses, autologous thyrocytes were severely damaged in 4 out of 6 cases and displayed moderate damage in the remaining 2 cases. The mean grade (±SE) of damage to the thyrocyte monolayer for this group was 3.7± 0.2. In comparison, lymphocytes from foetuses which had only been thyroidectomized produced only moderate damage in 3 out of 6 cases and slight destruction in the remainder by day 5 of cultivation. The mean grade of damage to thyrocyte monolayers in this group was 2.5±0.2. This was significantly lower ($P<0.01$) than the grade of damage to monolayers exposed to lymphocytes from thyroidectomized and thymectomized foetuses. Severe damage to thyrocyte monolayers occurred in all cases of both groups by 10 days of incubation (Table 23).

5.5. Discussion

The original demonstrations (McCullagh, 1970; Gershon and Kondo, 1971) of negative regulatory cells did not relate to autoimmunity, but to artificially induced tolerance. There have, nevertheless, been a number of reports of the appearance of suppressor cells in response to overt autoimmune reactions. Penhale *et al.* (1973, 1976) found that neonatal thymectomy could predispose to autoimmune thyroiditis in rats.

The transfer of normal syngeneic thymus cells into thymectomized animals inhibited the induction of this autoimmune thyroiditis. Their results suggested that suppressor cells could be one of the main factors in resisting induction of autoimmune responses in normal individuals. McCullagh (1990) showed that curtailment of thyroiditis occurred in thyroid tissue implanted in DA rats, which had experienced interference with development of self tolerance, as a result of inoculation of ^{131}I *in utero,* provided they were parabiosed with normal DA rats in adult life. These results suggest that normal DA rats possess migratory cells with the capacity to curtail expression of anti-thyroid reactivity by rats in which thyroid development has been disrupted before the development of immunocompetence.

In the present experiments, cultivated thyrocyte monolayers from a thyroidectomized foetal lamb retained an intact thyroid follicle structure after culture with lymph node cells from its normal identical foetal co-twin, whereas these thyrocytes sustained severe damage after incubation with autologous lymph node cells from the thyroidectomized co-twin itself. These distinctive *in vitro* responses to thyrocytes of lymphocytes from normal and thyroidectomized identical twin foetuses provided an opportunity to examine the possibility of the existence of an immune suppressor capacity in the normal co-twin. When lymphocytes from the two twins were placed together on the thyrocyte monolayer, the cytotoxic response of lymphocytes from the thyroidectomized co-twin was curtailed. Instead of severe damage to the monolayer, there were only some foci of slight or moderate destruction. This result implied that some suppressor (or negative regulatory) effect on anti-thyrocyte autoimmune responses could be exerted by lymphocytes from the normal co-twin.

The result presented in 5.2.2. showed that the curtailment of autoimmune cytotoxic responses by lymphocytes from normal animals also occurred in relation to anti-thyroid reactivity in DA rats. Demonstration of anti-autoimmune suppressor activity in normal lymphocyte populations from two widely separated species increased the likelihood that the regulatory process under observation was of wide applicability.

The occurrence of curtailment of autoimmune responses was influenced by the relative numbers of lymphocytes from normal and thyroid non-tolerant individuals. The

curtailment of autoimmune responses occurred when a ratio of 5:7 was established between whole lymphocyte populations from thyroidectomized and intact co-twin foetuses. When the two cell populations were mixed in equal numbers, there was no curtailment in about half of the cases tested. FACS separation of normal lymphocytes into subpopulations revealed that $CD8^+$ cells produced effective suppression of autoreactive lymphocytes at a lower ratio.

Curtailment of autoreactivity by normal lymphocytes was influenced by the sequence in which cells were introduced to thyrocyte monolayers. If autoreactive and normal lymphocytes were introduced simultaneously, suppression was not achieved as readily as when normal lymphocytes were placed on the monolayer some time before autoreactive cells. This raised the possibility that the activity of suppressor cells may have been mediated via an interaction with the potential target cells rather than by direct interaction with autoreactive lymphocytes.

The observed influence of the sequence of lymphocyte addition to thyrocyte monolayers provided some clues to mechanisms of regulation. A possibility could be that cells from the normal animal recognised the target autologous antigens, but with a much lower "affinity" than do autoreactive cells from thyroid non-tolerant animals. Perhaps the "low-affinity" cells engaged or bound with target autoantigens in such a way as to impair access by cytotoxic autoreactive cells or previous interaction of suppressor cells with target cells interfered with later engagement of cytotoxic cells with these targets. Another possibility might be that suppressor cells exerted a suppressive effect through secreting anti-idiotype antibodies to form an "idiotypic network". This process could require sufficient time in order for the suppressor cells to secrete antibodies.

Examination of the phenotype of T suppressor cells in Chapter 5.3. showed that a $CD8^+$ subset was responsible for the suppressor effect. To identify further the suppressor cell subset, a CD4 depleted population, which included $CD8^+$ cells, has been tested. This subpopulation failed to curtail autoimmune responses. The main difference between the two types of population was that double positive T cells ($CD4^+CD8^+$ subset) were included in the $CD8^+$population, but not in the CD4 depleted population.

This result suggested that the suppressor effect produced by the $CD8^+$ subset might require the assistance of $CD4^+CD8^+$ cells. There have been a few reports on the function of such double positive T cells. Sercarz and Krzych (1991) suggested that since T suppressor cells could be activated only by $Fc(C_H2\text{-}C_H3)$ domains of IgG, and not by the T help inducing C_H3 domain, $CD4^+CD8^+$ cells which were activated by $Fc(C_H2\text{-}C_H3)$ domains might be suppressor cells. It was unclear whether double positive cells would play a role as inducer cells or effector cells.

In this experiment, monoclonal antibodies have been utilised to label lymphocytes in order to identify cell subsets. However, there have been reports that monoclonal antibody could inhibit lymphocyte function. O'Rourke and Mescher (1993) suggested that anti-CD8 antibody could interfere with the cytolytic function of $CD8^+$ cells. However, Nabozny *et al.* (1991) found that anti-CD4 monoclonal antibody failed to inhibit the function of $CD4^+$ suppressor cells. This was evident in the continued induction of resistance to experimental autoimmune thyroiditis in mice given the antibody 24 hours before stimulation with mouse thyroglobulin. The continued induction of suppressor cells could not be explained on the basis of cessation of any interference of the monoclonal antibody with $CD4^+$ cells as the antibody remained demonstrable in the circulation for at least 14 days. In the present experiment, anti-CD8 monoclonal antibody did not inhibit the function of CD8 suppressor cells.

The experimental results in this Chapter and in Chapter 4.7 revealed that both cytotoxic and regulatory cells were from the $CD8^+$ lineage. One possibility could be that, though suppressor and autoreactive cells belong to same subset of lymphocytes, they possess additional cell surface antigens resulting in their distribution into different functional groups (Morimoto *et al.* 1985). Another possibility is that the ability of cytotoxic T lymphocytes to mediate cytolytic function or non-cytolytic suppressor function was dependent on their stage of maturation or activation (Pereira *et al.* 1988).

The results in Chapter 5.4. showed that thymectomy did not interfere with development of autoimmune responses. On the contrary, the occurrence of autoimmune responses was more marked at an earlier time when lymphocytes from thyroidectomized *and* thymectomized foetuses were tested. This suggested that the

thymus could play a role in negative regulation of autoimmune responses initiated by peripheral antigens. A possible role would be the production of suppressor cells against thyroid-reactive cytotoxic cells, or of their precursors in the thymus. This possibility was supported by the results (Chapter 5.3) that specific suppressor cells belonged to a thymus-derived lineage. Another possibility is that the thymus could eliminate anti-thyroid autoreactive cells even though thyroid specific antigens would not be expected to be available in the thymus normally. Thymectomy could lead to a relative excess of autoreactive cells.

CHAPTER 6: CELL-MEDIATED MECHANISMS IN THE INDUCTION OF AUTOIMMUNE RESPONSES AGAINST CULTIVATED THYROCYTES

6.1. Introduction

Stimulation of T cells leading to the induction of autoimmune responses is dependent upon interaction of major histocompatibility complex (MHC) bound antigen with T cell receptors. According to the conventional view, immunogens are presented by bone marrow-derived lymphocytes in lymphoid tissues on primary exposure to an antigen. In secondary responses, memory T cells may be distributed in non-lymphoid tissues and reactivated by antigen on different types of cells at peripheral sites (Knight, 1993). These mechanisms have been defined in the course of study of bacterial and viral antigens. However, it remains an open question whether the process of auto-immunogen recognition is the same as that operating during recognition of other types of antigens. Some self-antigens, especially those expressed on lymphoid cells, may be able to stimulate T cells in the same manner. Nevertheless, many organ-specific antigens, e. g. pancreatic islet cell antigens and major thyroid cell antigens, are expressed only on non-lymphoid cells or peripheral tissues and are not secreted in large amounts, if at all, into the bloodstream (Lo, 1992). These tissue-specific antigens are of great importance in explaining self-tolerance, since many of the major failures of tolerance to self, resulting in autoimmune diseases, are attributable to immune responses directed against them. As the process by means of which self-tolerance to such peripheral antigens is induced remains quite uncertain, the way in which autoimmune responses against peripheral self-antigens are initiated, and especially the role of the MHC in this process, has been much debated.

Posselt *et al.* (1992) proposed that peripheral antigens expressed on non-lymphoid cells that could not produce MHC molecules, and therefore could not present self peptides to T cells would not be tolerated by the immune system. Additionally, it is possible that some self-antigens could be present on cell membranes at such a low density as to be ignored by specifically reactive T cells. Posselt *et al.* (1992) injected pancreatic islet cells directly into the thymus of neonatal BioBreeding (BB) rats, a strain

which spontaneously develops autoimmune insulitis mediated by islet-specific T cells. After islet cell injection, the autoimmune diabetes, which would otherwise have developed, was prevented. This suggested that tissue-specific autoantigens on pancreatic islet cells did not gain access to the thymus in the normal course of events and so were not recognised there.

In contrast with the preceding findings, several investigators have reported the existence of peripheral self-antigen recognition and of recognition restricted by MHC. Lo *et al.* (1989) developed an animal model in which a transgenic class II antigen was expressed exclusively in non-lymphoid tissues of mice. The outcome was that tolerance developed to this antigen and spontaneous autoimmune disease did not develop. This result also implied that self antigens may be capable of inducing tolerance in T cells by associating with class II MHC antigens on non-lymphoid cells.

However, none of these experimental results has provided a clear indication of whether tolerance was directed to the MHC alone or to MHC plus peripheral antigens. In addition, it is doubtful whether the induction of peripheral specific antigen tolerance entails processes identical with those involved in the induction of tolerance to MHC, artificially expressed. To explore the extent of participation by peripherally expressed antigens in the induction of tolerance, McCullagh (1991) examined foetal lambs that had been submitted to thyroidectomy before the acquisition of immune competence, immediately followed by the implantation of a thyroid allograft from a donor of similar age. Thyroidectomy alone at this age leads to failure to develop self tolerance to thyroid tissues and to the appearance of specific autoimmunity. It was found that, whereas heavy lymphocytic infiltration appeared in reimplanted autografts, the primary thyroid allografts had not been rejected. These results strongly suggested that mechanisms exist for the peripheral recognition and induction of tolerance to specific antigens plus MHC, but not to the antigens alone.

On the other hand, the expression of MHC class II antigen on non-lymphoid cells has been proposed as a pathogenic mechanism for some organ-specific autoimmune diseases, *e.g.* autoimmune thyroiditis (Bottazzo, 1983). Pujol-Borrell *et al.* (1983) reported the expression of Class II antigen on the surface of thyroid cells in

human autoimmune thyroiditis, both Graves' and Hashimoto's disease. They cultured thyroid cells from these patients with phytohemagglutinin and then examined them for expression of MHC class II antigen by means of indirect immunofluorescence staining. Positive expression of MHC class II antigen was detected on thyrocytes from these patients, but not on normal human thyrocytes. Hence, Bottazzo et al. (1983) proposed that class II antigen abnormally expressed on non-lymphocytes might enhance the presentation of autoantigen on the cell surface. As a result of this, it was suggested, that T cells could be stimulated leading to induction of autoimmune responses. They also suggested that viral infection, with secretion of IFN-γ, induced the expression of MHC class II antigen, because IFN-γ was shown to be able to induce class II expression on epithelial cells (Streeg *et al.* 1982).

A contrary viewpoint has been presented recently by Hamilton *et al.* (1991), namely that aberrant class II MHC expression by thyroid epithelium cells in established thyroid autoimmune disease could be the ***result*** of the release of IFN-γ by adjacent lymphocytes subject to autoimmune attack. They found that the expression of thyrocyte MHC class II in a group of Hashimoto's and Graves' disease patients usually occurred adjacent to IFN-γ positive lymphocytes in stained sections. Both expression of class II antigen and positive staining for IFN-γ appeared in areas containing large numbers of infiltrating lymphocytes. They explained their results on the basis that lymphocytes within the thyroid could release IFN-γ and evoke subsequent expression of class II MHC antigen on the thyroid cells.

In this chapter, I will examine whether recognition of peripheral self-antigens is restricted by MHC. For this purpose, bilaterally thyroidectomized foetal lambs have received thyroid allografts at 51-54 days of gestation. Donors were foetal lambs of similar gestational age. Excised thyroids were converted into cell suspensions and stored at -196°C. At 100-120 days gestation, lymph nodes were removed from the donor and the recipient and lymphocyte suspensions were prepared. Freshly isolated lymphocytes were then cultured with monolayers of either donor or recipient thyrocytes that had been established in culture 3 days previously (Figure 30). These experiments were designed to define conditions under which resistance to the development of

autoimmune responses against cultivated autologous thyrocytes could be achieved following allothyroid implantation in the thyroidectomized foetus.

The significance of expression of class II antigens on cultivated thyrocytes that have been incubated with autologous lymphocytes from anti-thyroid autoreactive rats has also been examined.

6.2. Examination of the role of the MHC in the recognition of peripheral antigens during autoimmune responses against cultivated thyrocytes

Four types of procedure were undertaken. In the first, lymphocytes from normal foetuses were incubated with allogeneic thyrocyte monolayers. In the second and third, lymphocytes from thyroidectomized foetuses that had received thyroid allografts were tested on thyrocyte monolayers derived respectively from thyroid allograft recipients and donors. Finally, the reactivity of lymphocytes from thyroidectomized foetuses, in receipt of allogeneic thyroid implants, was tested on third party thyrocytes.

6.2.1. *Cytotoxicity of lymphocytes from normal foetal lambs for cultivated thyrocytes from allogeneic foetal lambs*

When lymph node cells from normal foetal lambs were introduced to cultivated thyrocytes from allogeneic foetal lambs, severe destruction of thyrocytes was observed by 10 days of coculture in all of the 7 cases tested (Table 24). Cloudly swelling, hyaline-like degeneration and lysis, especially at the apices of epithelial thyrocytes, was followed by widespread follicular destruction. The mean degree of cytotoxicity was 4.3 ±0.2. There was no noticeable morphologic difference between damage to thyrocyte monolayers resulting from alloaggression and autoreactivity (as described in Chapter 4.2).

6.2.2. *Cytotoxicity for cultivated autologous thyrocytes of lymphocytes from foetal lambs that have previously been submitted to thyroidectomy and allogeneic thyroid implantation*

Lymphocytes from 11 foetuses were tested. In 10 out of 11, severe destruction of cultivated autologous thyrocytes was observed by 10 days of coculture. Follicle epithelial cells showed cloudly swelling, followed by hyaline-like degeneration and lysis. Most follicular structures disappeared to be replaced by single, deformed, and degenerate follicles. The mean degree of cytotoxicity was 4.5±0.2, if foetus W18 was excluded. Thyrocytes from the W18 foetus retained normal follicle structure with only slight damage observed. Significantly, in this case, residues of recipient thyroid gland were found at *post mortem* (Table 25 and Figure 31).

6.2.3. *Cytotoxicity of lymphocytes from thyroidectomized foetal lambs, in receipt of allogeneic thyroid grafts, for cultivated thyrocytes from the thyroid allograft donor*

Nine cases have been tested. In 6 of these, normal follicle structures were preserved with only foci of slight damage to the thyroid monolayer after 10 days of coculture (Table 26 and Figure 31). In another 2 out of the 9 cases (allograft recipients Y296, W218) there was moderate damage of the cultivated thyrocytes. Nevertheless, in these two cases, most follicle epithelial cells retained their distinct structure. Foci of degeneration of thyroid follicles were occasionally found. Only one out of 9 cases developed severe thyroid monolayer damage. This was allograft recipient Or 992, . In the case of foetuses Y296 and Or 992, there was no macroscopic evidence of implanted thyroid when examined at *post mortem.* The mean grade of damage to thyrocyte monolayers from thyroid graft donors was 2.1±0.4.

6.2.4. *Cytotoxicity of lymphocytes from thyroidectomized-allogeneic-thyroid implanted foetal lambs for cultivated thyrocytes from other (third party) allogeneic foetal lambs*

Six cases have been tested. Cultivated thyroid monolayers were severely damaged in all cases after incubation with lymphocytes from thyroidectomized-thyroid-allograft-implanted foetal lambs for 10 days (Table 27). Most thyrocyte follicles had been subject to degeneration and lysis. The mean grade of cytotoxicity was 4.2±0.3.

6.3. The significance of MHC class II antigen expression by rat thyrocyte monolayers in the development of an autoimmune response against cultivated thyrocytes

The capacity to induce class II antigen expression on syngeneic thyrocyte monolayers of lymphocytes from 10 ^{131}I exposed DA rats, that had not received renal subcapsular grafts of syngeneic thyroid and of lymphocytes from 10 normal rats was compared. After incubation with lymphocytes from ^{131}I exposed rats for 48 hours, a mean of 12.9%±3.1% thyrocytes (for all 10 ^{131}I exposed cases tested) manifested positive staining for class II antigen. In contrast, after incubation with normal rat lymphocytes, a mean of only 0.8%±0.5 thyrocytes (for all 10 normal cases tested) displayed positive staining (Table 28 and Figure 32). Thyrocytes cultured alone were completely negative when tested for staining for class II antigen in all 10 cases.

Thyrocyte monolayers exposed to lymphocytes from 3 of the ^{131}I exposed rats failed to show positive staining. In the case of one of these (1/7), not only did the thyrocyte monolayer not stain positively for class II antigen, but it was not subject to cytotoxic changes when examined morphologically after 10 days of coculture with lymphocytes. In the case of ^{131}I exposed rats 1/9 and 2/9, staining for class II antigen was not undertaken until the third and fifth days of coculture respectively.

Positive expression of class II antigen on thyrocytes was influenced by the duration for which they had been cultivated with lymphocytes from syngeneic ^{131}I exposed rats. In 6 cases examined after 24 hours of coculture, a mean of 17.5% thyrocytes showed positive staining of class II antigen. A mean of 12% thyrocytes manifested positive staining at 48 hours of coculture in the 4 cases examined after this interval. No positive staining of thyrocyte monolayers was observed in 2 cases examined after 72 hours of coculture, nor in any of 10 others examined after 120 hours of coculture (Table 29).

6.4. Deficiency of histone 3 in culture medium of lymphocytes from ^{131}I exposed DA rats or thyroidectomized foetal lambs

Comparison of the supernatant from normal and autoreactive lymphocytes, cultured either alone or with a thyroid monolayer, revealed a consistent difference. Supernatants collected after 5 days culture of lymph node cells from 6 normal rats and 6 ^{131}I exposed rats, and also those collected after co-cultivation of these cells with syngeneic thyrocytes, have been examined by SDS-PAGE. A protein band, around 17.6kD was lacking from the supernatant of the lymph node cells from ^{131}I exposed rats (Figure 33). This band was invariably observed in the supernatant from normal lymph node cells, from normal lymph node cells incubated with syngeneic thyrocytes, and from mixtures of normal lymph node cells with lymph node cells from ^{131}I exposed rats incubated with syngeneic thyrocytes. This band could not be clearly observed in the supernatant from cultivated lymph node cells of ^{131}I exposed rats, or in supernatant from the incubation of these cells with syngeneic thyrocytes. This band was not observed in supernatants from thyrocytes cultivated alone. In addition, supernatants of lymphocytes from different organs of normal rats have been examined. This band could be clearly observed in the supernatant from normal thymus and lymph node cells, whereas it was lacking from the supernatant from normal spleen cells (Figure 34).

A band at 17.6 kD was also lacking from the supernatant of lymph node cells from all 4 thyroidectomized foetal lambs examined, when incubated alone, and from the supernatant of these cells when cultivated with autologous thyrocytes. Nevertheless, this band was clearly evident in the supernatant from lymph node cells of 4 hemi-thyroidectomized foetal lambs cultivated alone.

This protein band that was lacking from the supernatant of autoreactive lymphocytes could be found in the supernatant of lymph node cells from normal foetal lambs. A substantial quantity of this protein was prepared by culturing many batches of normal rat lymph node cells, running SDS-PAGE, and transferring the band to a PVDF membrane by electroblotting. The protein sequence of this band was analysed by a model 470 protein sequenator. Ten amino acids have been recognised. They were Ala(A), Arg(R), Thr(T), Lys(K), Gln(Q), Thr(T), Ala(A), Arg(R), Lys(K), and Ser(S). The relative frequency of these 10 amino acids was the same as that of histone 3 from other species analysed by computer. However, the complete amino acid sequences of

histone 3 from rats or sheep have not been reported. Consequently, it is not possible to make this identification with complete certainty.

6.5. Discussion

The experiments reported in this Chapter dealt with the influence of histocompatibility determinants expressed by potential target cells on their susceptibility to autoimmune processes.

To investigate the possibility of operation of histocompatibility restriction in relation to immune recognition of organ-specific antigens, four combinations of lymphocytes and thyrocytes were examined. Lymphocytes from normal foetal lambs reacted against allogeneic thyrocyte monolayers producing severe damage. Lymphocytes from foetuses which had been submitted to thyroidectomy at 54 days gestation and had then received a thyroid allograft were tested against thyrocyte monolayers from three sources. Whereas thyrocytes from the thyroidectomized and allografted foetal donor itself, and also thyrocytes from a third party foetus, were severely damaged by these lymphocytes, thyrocyte monolayers derived from the thyroid allograft donor were spared. Whereas normal thyrocytes do not express class II histocompatibility antigens, these were detectable during the first 48 hours of exposure to autoreactive lymphocytes.

In attempting to define the influence on presentation of organ specific autoantigens of histocompatibility determinants on the thyrocyte surface, it would be anticipated that antigenic peptides would complex with class I molecules for interaction with $CD8^+$ lymphocytes. The capacity of lymphocytes from normal foetuses to react against allogeneic thyrocyte monolayers provided a clear indication that the target cells expressed adequate levels of histocompatibility determinants to achieve allo-sensitization. The earlier demonstration (in Chapter 5.2.1) that lymphocytes from normal foetuses did not react against monolayers of thyrocytes from their identical twins effectively excluded the possibility of *de novo* autosensitization of lymphocytes in culture. No distinction could be drawn, on the grounds of the morphology of the

damage, nor of its *tempo*, between reactivity directed against allo- and auto-antigens on thyrocytes.

The failure of lymphocytes from thyroidectomized and thyroid-allografted foetal lambs to become tolerant towards autologous thyrocytes demonstrated that the process of induction of self tolerance, at least in relation to thyroid-specific antigens, is histocompatibility restricted. That is, the antigenic peptides derived from thyroid autoantigens are incapable of inducing tolerance unless presented on class I MHC determinants corresponding to those expressed by the affected lymphocytes. The improbability of either expression in, or entry to, the thymus of the foetal lamb by thyroid-specific antigens before immunological maturation commences strongly suggests that self-tolerance of them is a post-thymic phenomenon. The experiments reported in Chapter 5.3.5 in which $CD8^+$ lymphocytes from normal foetuses were shown to block anti-thyroid autoreactivity of lymphocytes from their thyroidectomized identical twins indicated that this phenomenon was predominantly based on suppression. Consequently, it may be inferred from the experiment under discussion at present that histocompatibility restriction was operating at the level of induction of T suppressor cells. It would appear that this process requires presentation of antigen by histocompatible cells. Re-processing of thyroid-specific antigen released from the allografted thyroid gland and its subsequent presentation by host phagocytic cells (if it occurred) was not adequate for suppressor induction.

The absence of cytotoxic damage to thyrocyte monolayers derived from thyroid allograft donors on exposure to lymphocytes from the thyroidectomized recipients of those allografts correlates with the retention of the allografts themselves, albeit sometimes with lymphocytic infiltration. Both outcomes implied that substantial allograft tolerance had been induced by the allograft. However, preservation of these thyrocyte monolayers contrasts with the demonstrated capacity of the same lymphocyte population to attack autologous monolayers. Apparently, concurrent allograft tolerance precluded autoreactivity against target cells expressing tolerated determinants despite the presence of lymphocytes reactive against thyroid-specific antigens. In the absence of any clear indication of the mechanism underlying allograft tolerance induced by thyroid

implants, namely whether clonal deletion or anergy had occurred or suppressor cells had been induced, it is not feasible to propose a process whereby this tolerance interfered with autoreactivity against the tolerated target cells.

Attack upon third party, allogeneic thyrocyte monolayers by lymphocytes from thyroidectomized and thyroid allografted foetuses could be attributable to either, or both, of autoreactivity against thyroid-specific antigens or alloreactivity. This result, nevertheless, served to establish the specific nature of the unreactivity observed on the part of the same population of lymphocytes when cultivated on monolayers from the relevant thyroid allograft donor.

The thyrocytes from one thyroid allograft donor sustained severe damage and two others sustained moderate damage following incubation with the lymphocytes from the thyroidectomized recipient of the thyroid allograft derived from them. In two of these 3 cases (Y296 and Or992) failure to induce unreactivity was attributed to failure of survival of the thyroid allograft, detected at *post mortem.* In the third case, the thyroid implant was found at post mortem examination but lymphocytes from this foetus nevertheless were moderately reactive against donor thyrocytes. No simple explanation for this discrepancy was apparent.

Expression of class II MHC antigens by thyrocytes has often been reported in the course of autoimmune thyroiditis. The relationship underlying this association (whether expression of class II antigens contributes to, or is a consequence of, expression of autoreactivity against the cells expressing them) has been contested. The results in Chapter 6.3. showed that cultivated thyrocytes expressed class II antigen after incubation with lymphocytes from ^{131}I exposed rats, while thyrocytes cultivated alone or thyrocytes incubated with normal syngeneic lymphocytes did not express class II antigen. These results suggested that class II antigen expression on cultivated thyrocytes was related to autoimmune responses against thyrocyte antigens, but was only secondary to or a *result* of presenting lymphocytes from rats non-tolerant to thyroid self-antigens. This consequence supported the viewpoint proposed by Hamilton *et al.* (1991), namely that MHC class II antigen expression on the thyrocytes could be the result of the release of interferon-gamma (IFN-γ) by infiltrating lymphocytes in

adjacent thyroid tissue. The expression of class II antigens only for the first 2 to 3 days after introduction of lymphocytes into the thyrocyte culture would implied that IFN-γ release occurred only for a limited period. On the other hand, it is unlikely that class II expression plays a primary role in the cytotoxicity observed in thyrocytes because $CD8^+$ population, which appears to be responsible for autoimmune responses in present experiments is stimulated restrictively by specific antigens associated with MHC class I antigen, but not with MHC class II antigen (Julius *et al.* 1993).

The result in Chapter 6.4. showed that there was a deficiency of a H3 component in the culture supernatant of lymphocytes from ^{131}I exposed rats or thyroidectomized foetal lambs in comparison with supernatant from normal lymphocytes. This H3 protein band could be clearly observed in the supernatant of lymphocytes from normal rats cultivated alone or from hemi-thyroidectomized foetal lambs. It could also be found in the supernatant of normal lymphocytes cultivated with thyrocytes. This protein band was not detected in the supernatant from thyrocytes cultivated alone or in culture medium alone. By comparing the supernatants from cultivated lymphocytes collected from different organs of DA rats, it has been found that culture supernatants of spleen cells lacked this protein band, whereas it could be observed in the supernatant from thymus or lymph node cells. It is likely that those lymphocytes which possess potential autoreactivity (including normal spleen cells) do not release of H3.

CHAPTER 7: GENERAL DISCUSSION

While major issues concerning the experimental results in this thesis have been extensively discussed in Chapter 3, 4, 5 and 6, several general subjects require some further consideration.

To recapitulate the principal experimental findings, it was demonstrated that a protocol which had previously been shown to induce experimental autoimmune thyroiditis in foetal lambs and rats, namely interference with development of, the thyroid gland before immunological maturation had commenced and its subsequent reimplantation, could be adapted for investigation *in vitro.* This was achieved by introducing lymphocytes from foetal lambs or DA rats, prepared as previously, onto autologous thyrocyte monolayers. The establishment of this *in vitro* approach to examination of experimental autoimmune thyroiditis in thyroid-ablated animals that have failed to become tolerant of thyroid self-antigens afforded opportunities to explore the mechanisms of resistance to induction of abnormal autoimmune responses in <u>*normal*</u> individuals. This investigation was based on testing of the capacity of lymphocytes from normal animals to interfere with the expression of cytotoxicity against thyrocytes by autoimmune lymphocytes.

Lymphocytes from thyroidectomized foetal lambs, and from DA rats exposed to ^{131}I *in utero,* regularly produced damage on autologous thyrocyte monolayers whereas cells from normal identical co-twin lambs and normal syngeneic rats were without effect on thyrocytes. Curtailment of autoimmune responses by lymphocytes from thyroid deprived animals was observed when mixtures of lymphocytes from autoimmune and normal individuals of both species were placed on autologous thyrocyte monolayers. These results indicated that lymphocytes from normal individuals possessed a capacity for negative regulation of anti-thyroid autoimmune responses. The appearance of suppressor cells in normal <u>*foetal*</u> lambs indicated that they had been produced during the period of establishment of self tolerance. It suggested that such suppressor cells may be of importance in normal resistance to spontaneous autoimmunity.

The implantation of an allogeneic thyroid in a thyroidectomized foetal lamb failed to prevent induction of autoimmune responses by the recipient's lymphocytes against cultivated autologous thyrocytes. However, implantation of allogeneic thyroid in a thyroidectomized foetal lamb induced tolerance of the recipient's lymphocytes for cultivated donor thyrocytes. These results were relevant to understanding the process of recognition of peripheral antigens during the establishment of self-tolerance. During this process in thyroid allografted lambs, immunocomponent cells (including suppressor cells) are required to recognise not only thyroid antigens alone, but also donor MHC expressed on cells within the allograft.

Three general issues will be considered in this chapter. The first relates to the significance of the various parameters available to assess the course of *in vitro* experimental autoimmune thyroiditis induced by interference with self-tolerance to thyroid antigens in foetal life. The second issue concerns the mechanism of negative regulation of autoimmune responses in normal individuals. The third pertains to pathogenic mechanisms of autoantigen recognition during the induction of abnormal autoimmune responses.

To assess the presence of experimental autoimmune thyroiditis *in vitro*, establishment of optimum culture conditions for differentiation of target thyroid antigen cells from foetal lambs or neonatal DA rats was a pre-requisite so that self-antigens normally expressed by these cells would be retained. Failure of cultivated thyrocytes to express determinants which were the target of autoreactivity could lead to false negative outcomes when lymphocytes from autoimmune donors were co-cultivated with them. As there was a requirement to maintain foetal lamb thyrocytes by means of cryopreservation for periods in excess of 8 weeks whilst immunological maturation of the donor was occurring, and as these cells had not yet differentiated sufficiently to undertake normal thyroid function at the time of their collection from the foetus, the ability of cryopreserved thyrocytes to differentiate after thawing required attention. The experimental results in Chapter 3. have shown that cryopreserved-thawed foetal lamb thyrocytes, differentiated normally, as did neonatal DA rat thyrocytes, when established in culture under suitable conditions. Among the various factors required for successful

culture were a high density of cultivated thyrocytes, which produced conditions favouring cell clustering and inclusion of TSH in the culture medium which played a crucial role in the maintenance of thyroid follicular structure and iodine uptake by cultivated thyrocytes. Both increasing the density of culture seeding and creating conditions favourable for cell clustering enhanced cell-cell contact and so promoted intercellular cooperation and communication. Inclusion of TSH in the culture medium is likely to have stimulated an increase of cyclic AMP concentration in the cultivated thyrocytes leading to the synthesis of specific RNA and the formation of proteins involved in intercellular recognition, aggregation and organisation of thyroid cells. The correlation between formation of follicular structure and enhancement of iodine uptake of thyrocytes following inclusion of TSH in the culture medium suggested that maintenance of the normal properties of cultivated thyrocytes depended on the formation and conservation of intact follicular structure by cultivated thyrocytes. The noticeable restriction of cytotoxic effects to those areas of the thyrocyte monolayers in which follicular structure was well established and the sparing of otherwise unorganised epithelial areas accorded with the expectation from morphology of restriction of the target antigens to the former areas.

The serum concentration of the culture medium also influenced the morphology of cultivated thyrocytes and, in consequence, is very likely to have influenced the expression of thyroid-specific antigens. High concentrations of serum (10%) induced rapid formation of follicles and overgrowth of fibroblast-like cells. Low concentrations of serum (<2.5%) restricted the formation of follicles and iodine uptake. Optimum culture conditions for conservation of follicular structure by cultivated thyrocytes entailed the establishment of a high density of cells in a small area of culture surface and the incorporation of TSH (20mU/ml) and FCS (5%) in the culture medium.

The two *in vitro* parameters of cell-mediated responses to be examined in experimental autoimmune thyroiditis were cytotoxic responses against target cells and proliferative responses by effector cells. Under the culture conditions which were developed, cytotoxic effects on cultivated thyrocytes in the present experiments were found to be a reliable representation of anti-thyroid autoimmunity as it occurred in whole

animals *in vivo*. However, there was no correlation between cytotoxic assays on monolayers and proliferative responses of lymphocytes. Positive proliferative responses were observed in lymph node cells from *normal* DA rats or foetal lambs after incubation with autologous thyrocytes, whereas negative cytotoxic responses were observed in these individuals. These results suggested that proliferative responses of effector lymphocytes, as assessed by [H^3]thymidine incorporation into cellular DNA in the present experiments, is a parameter lacking significance as an indication of autoreactivity.

The induction of anti-thyroid autoimmunity in foetal lambs and in DA rats following interference with development of self-tolerance to thyroid antigens strongly supported earlier experiments which indicated that exposure of peripheral self-antigens to the immune system was a basic requirement to ensure development of self tolerance (at least in the case of thyroid antigens). The observation that some foetal lambs that retained small amounts of thyroid tissues, nevertheless developed the capacity to attack an autologous thyrocyte monolayer suggested that the establishment of self-tolerance required exposure to a threshold level of organ-specific antigens. Lower levels of antigen presentation were not inconsistent with the development of autoimmunity.

Cytotoxic responses occurred more consistently and were more severe with lymphocytes from thyroidectomized foetal lambs than with cells from ^{131}I exposed DA rats. This could be a reflection of differences in the protocols employed to destroy thyroid tissue in foetal lambs and rats. In rats, damaged thyroid tissue was retained, although it appeared highly abnormal morphologically, in contrast with the complete removal of thyroid tissue from thyroidectomized foetal lambs. The development of anti-thyroid autoimmunity in rats, nevertheless, indicated that major thyroid antigens were not tolerated. On the other hand, some of the differences in intensity of cytotoxic responses may have been attributable to species differences.

It was consistently observed that cytotoxic responses produced by lymphocytes from ^{131}I exposed DA rats became more severe following pre-stimulation of lymphocytes by implantation of syngeneic thyroid tissue. A possible explanation for this could be that autoreactive lymphocytes had clonally expanded after *in vivo* stimulation by thyroid

antigens. This might amplify the cytotoxic effect of these lymphocytes when they encountered the same antigens *in vitro*.

Interpretation of anti-thyroid autoimmune reactivity in these experiments must take account of the possible influence of hypothyroidism on immune responsiveness. Thyroid ablation or destruction both induce hypothyroidism which has been demonstrated to retard development of the immune system. This would be expected to result in diminished immune reactivity in the present experiments. It would certainly not have augmented the development of the cytotoxic responses which were studied.

A second general issue raised by the present experiments concerned the operation of mechanisms for negative regulation of autoimmune responses in normal individuals. The occurrence of experimental autoimmune thyroiditis, without any requirement for deliberate immunisation, after interference with development of self-tolerance of thyroid antigens implies that the mechanisms normally responsible for self-tolerance may actively curtail the induction of abnormal autoimmune responses.

Demonstration of prevention of autoimmune reactions against thyrocytes by suppressor cells from normal foetal lambs and rats suggests that the observed phenomenon may have a broad applicability. It appears highly likely that the establishment of self-tolerance is not only a process of elimination, or inactivation, of autoreactive cells, but also of the formation of specific suppressor cells. As there are a number of reports suggesting that autoreactive cells can exist in normal individuals, it appears increasingly possible that suppressor cells play a crucial role in the induction and the maintenance of self-tolerance and are not a mere epiphenomenon. Any defect of suppressor cells or decrease in their numbers could be expected to facilitate the induction of autoimmune diseases.

The present experiments have not distinguished whether suppressor cell effects were induced by the target antigen directly or by a response to autoreactive thyrocytes. The former possibility may be the more likely. If suppressor cells were produced primarily in response to the autoreactive cells with the cytotoxic capacity of which they then interfere, their generation in the thyroid-depleted, autoimmune animals in this experiment might have been expected. On the other hand, if the stimulus to production of

suppressor cells is provided by presentation of self antigen at the appropriate early stage of development of the immune system, their absence from thyroidectomized animals would be anticipated. If suppressor cells are produced in response to exposure to antigen, it is likely that they would share some attributes with the cytotoxic cells (specifically reactive against that antigen) that they block.

As with their induction, alternative explanations exist for the mode of action of suppressor cells. These correspond with the alternative processes of induction. One possibility is that suppressor cells bind with target autoantigens in such a way as to impair access by cytotoxic autoreactive cells, that is, in some way previous interaction of suppressor cells with target cells interfered with later engagement of cytotoxic cells with the affected target cells. Another possibility is that suppressor cells exert their effect through an anti-idiotype reaction directed against the relevant autoimmune cells.

The observation that normal spleen lymphocytes mounted intensive cytotoxic responses against syngeneic thyrocytes, whereas these responses failed to occur when normal thymus or lymph node cells were used, requires explanation consistent with the preceding discussion. One possibility could be that numerous autoreactive cells persist in the spleen of normal individuals after establishment of self-tolerance. As indicated in Chapter 1, there have been reports of the presence of anti-thyroid autoimmune cells in normal individuals. The activities of these cells might be controlled or suppressed *in vivo* by suppressor cells from other sites in the lymphoid system. Under the altered conditions prevailing *in vitro,* however, it is possible that these cells might escape from suppressor influence leading to cytotoxic responses.

The response of those foetal lambs that were submitted to thymectomy in addition to thyroidectomy, namely augmentation of the *in vitro* occurrence of anti-thyroid autoimmune responses, was also consistent with the existence of mechanisms for negative regulation of autoimmunity. It is possible that the thymus generates antigenically non-specific suppressor cells that resist the induction of abnormal autoimmunity.

A third general issue concerned recognition of peripheral antigens and the MHC role in this recognition. It has been proposed that peripheral antigens expressed on non-lymphoid cells in a tissue such as the pancreatic islet would not be recognised by

immunocomponent cells during the stage of formation of self-tolerance. It was also unclear whether the recognition of peripheral antigens in post thymic tolerance was restricted by MHC antigens. The present experiments showed that implantation of a thyroid allograft in a thyroidectomized foetal lamb failed to prevent the expression of an autoimmune response against cultivated autologous thyrocytes in later life. Nevertheless a thyroid allograft induced tolerance on the part of the recipient's lymphocytes to cultivated thyrocytes from the donor. These results indicated that recognition of thyroid antigens (and perhaps also of other peripheral antigens) during the establishment of self-tolerance entailed the recognition of thyroid antigens in association with MHC, as occurs in induction of immunity. Tolerance induced by exposure of the immature immune system to thyroid antigens presented in association with the donor MHC was only evinced towards thyroid antigens when again presented in that context. These experiments suggest that thyroid-specific antigens derived from donor thyrocytes had been presented to the immature immune system by those thyrocytes rather than by host antigen presenting cells after destruction of transplanted thyrocytes.

In conclusion, the present studies principally explored negative regulation mechanisms of abnormal autoimmune responses against thyroid antigens. The existence of suppressor cells appeared to be one of the main elements in negative regulation mechanisms. The development of self-tolerance represents not only a process of elimination of autoreactive cells, but also one of formation of suppressor cells. One implication of the present experiments is that there could be a substantial contribution to the induction of autoimmune diseases by defects of suppressor cells. If so, stimulating the induction of suppressor cells could be an appropriate way to resist the occurrence of autoimmune diseases. This approach could imply a requirement for some modification of the conventional approach to treatment of autoimmune diseases. Conventional procedures to treat autoimmune disease have concentrated on inhibiting the function of autoreactive cells. There may be scope for strategies to augment the frequency and activity of the appropriate suppressor cells.

Figure 1. Morphological features of autoreactivity of lymphocytes against autologous thyrocyte monolayers.

(Fixation of the specimens in these photographs was undertaken 10 days after adding lymphocytes to thyrocyte monolayers. Coverslips bearing the indicated cells were all stained by means of H&E. Magnification ×117 unless otherwise stated.)

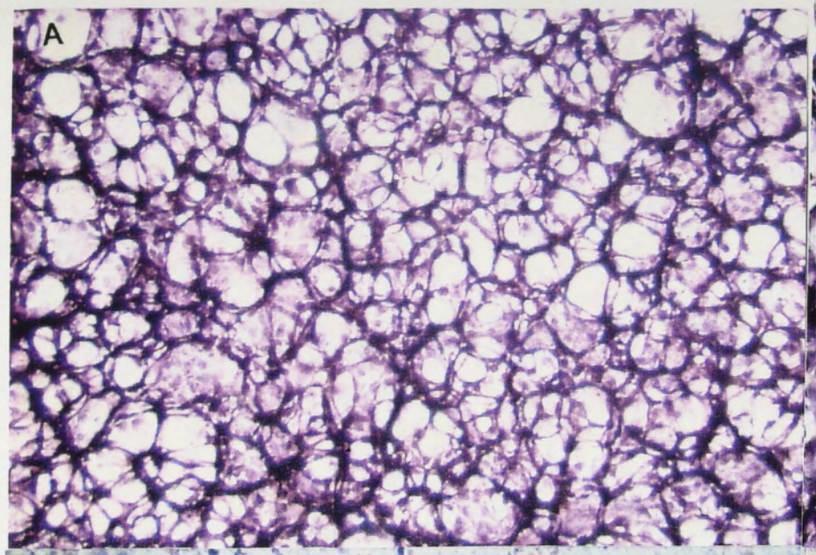
(A) *Normal foetal lamb thyroid monolayer in absence of lymphocytes.* The thyrocyte monolayer consisted of numerous intact thyroid follicles showing the distinctive structure of follicle epithelial cells. Most follicles were connected with each other. (Grade 0).

(B) *Slight damage to thyrocyte monolayer following addition of lymphocytes from normal foetal lamb.* (Magnification ×292). Thyrocyte follicles remained intact in most areas of cultivated thyrocyte monolayers. Thyrocyte atrophy (single arrow) and detachment from the coverslip surface (double arrows) were occasionally observed. (Grade 1).

(C) *Severe damage to thyrocyte monolayer following addition of lymphocytes from thyroidectomized foetal lamb.* Thyrocyte follicle cells displayed cloudly swelling, hyaline-like degeneration and lysis. Major damage was apparent at the apices of those epithelial thyrocytes which had organised into thyroid follicles. The interconnection of follicles was almost completely lost to be replaced by isolated, deformed, degenerated follicles. (Grade 4).

(D) *Severe damage to thyrocyte monolayer following addition of lymphocytes from thyroidectomized foetal lamb.* In severe cases, complete lysis of the thyrocyte monolayer has been observed. (Grade 5).

(E&F) *Detachment of thyrocyte monolayer following addition of excessive numbers lymphocytes from normal foetal lambs.* After incubation with 15×10^6 lymphocytes from a normal foetal lamb, the autologous thyrocyte monolayer displayed severe atrophy and detachment of numerous thyrocytes from coverslip surface. However, follicles remained intact, even when detached from the coverslip. (Mag.E=×47, F=×117)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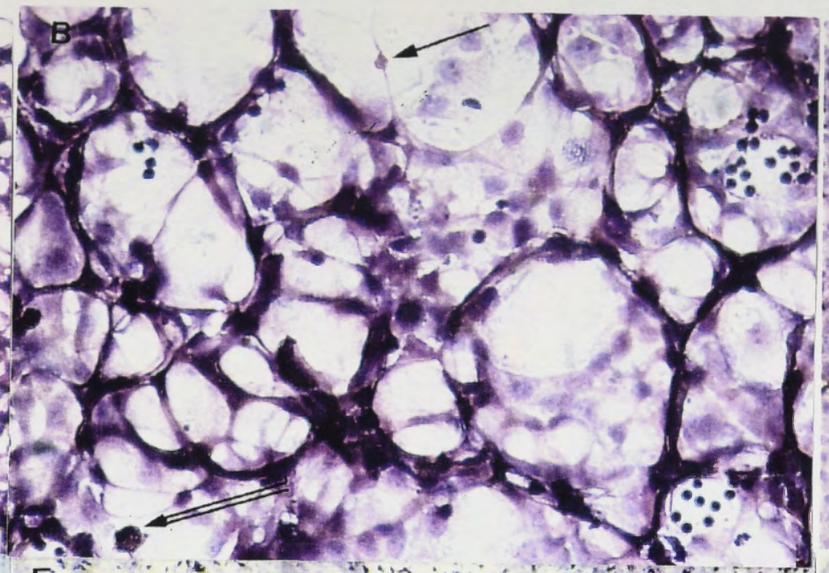
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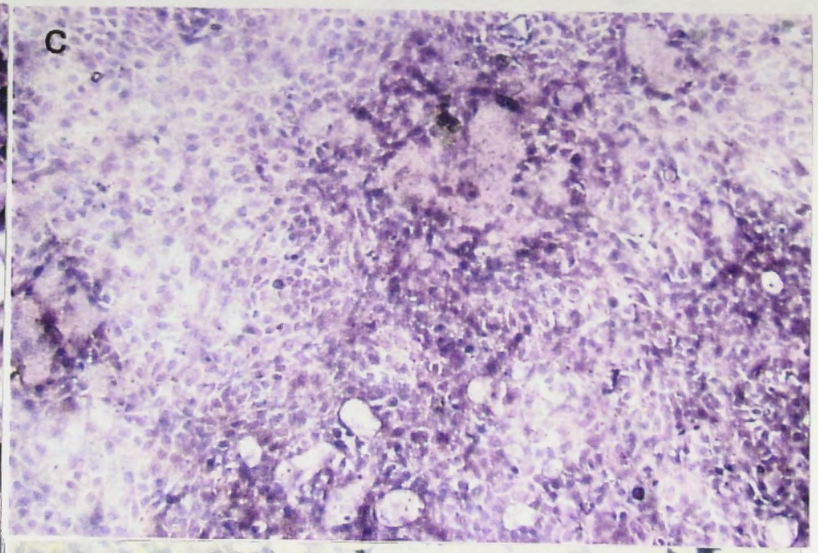
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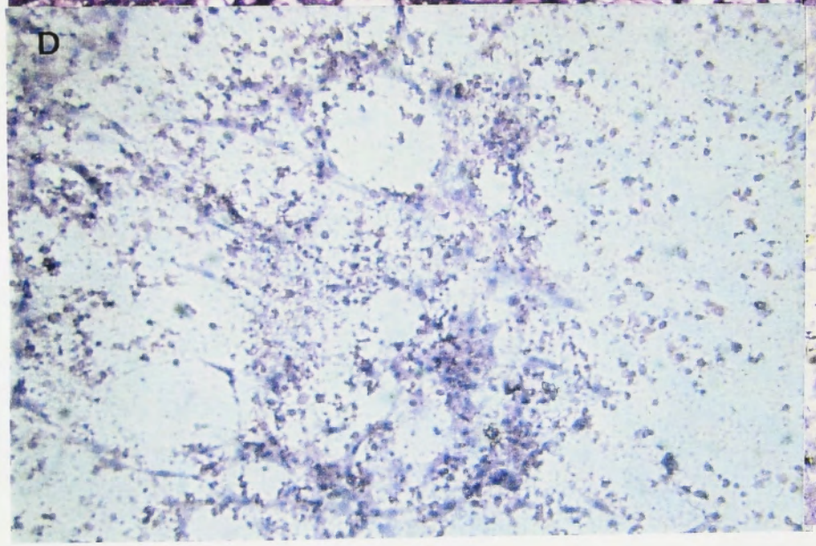
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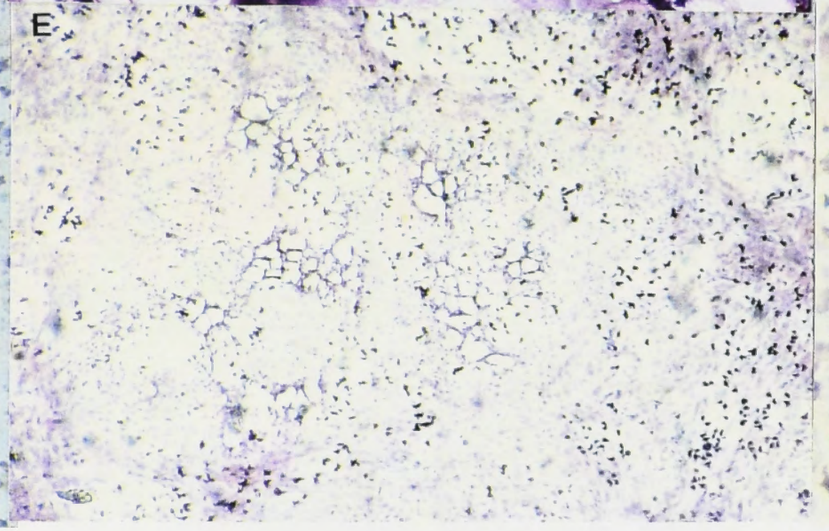
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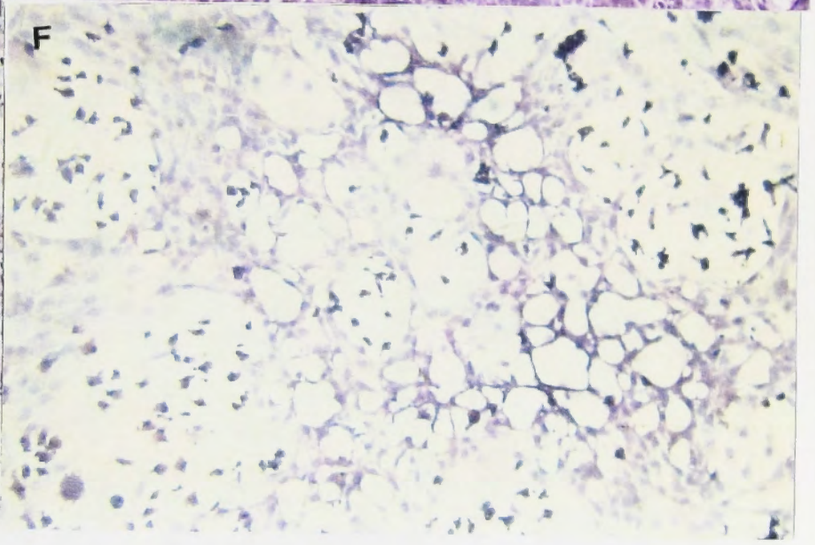
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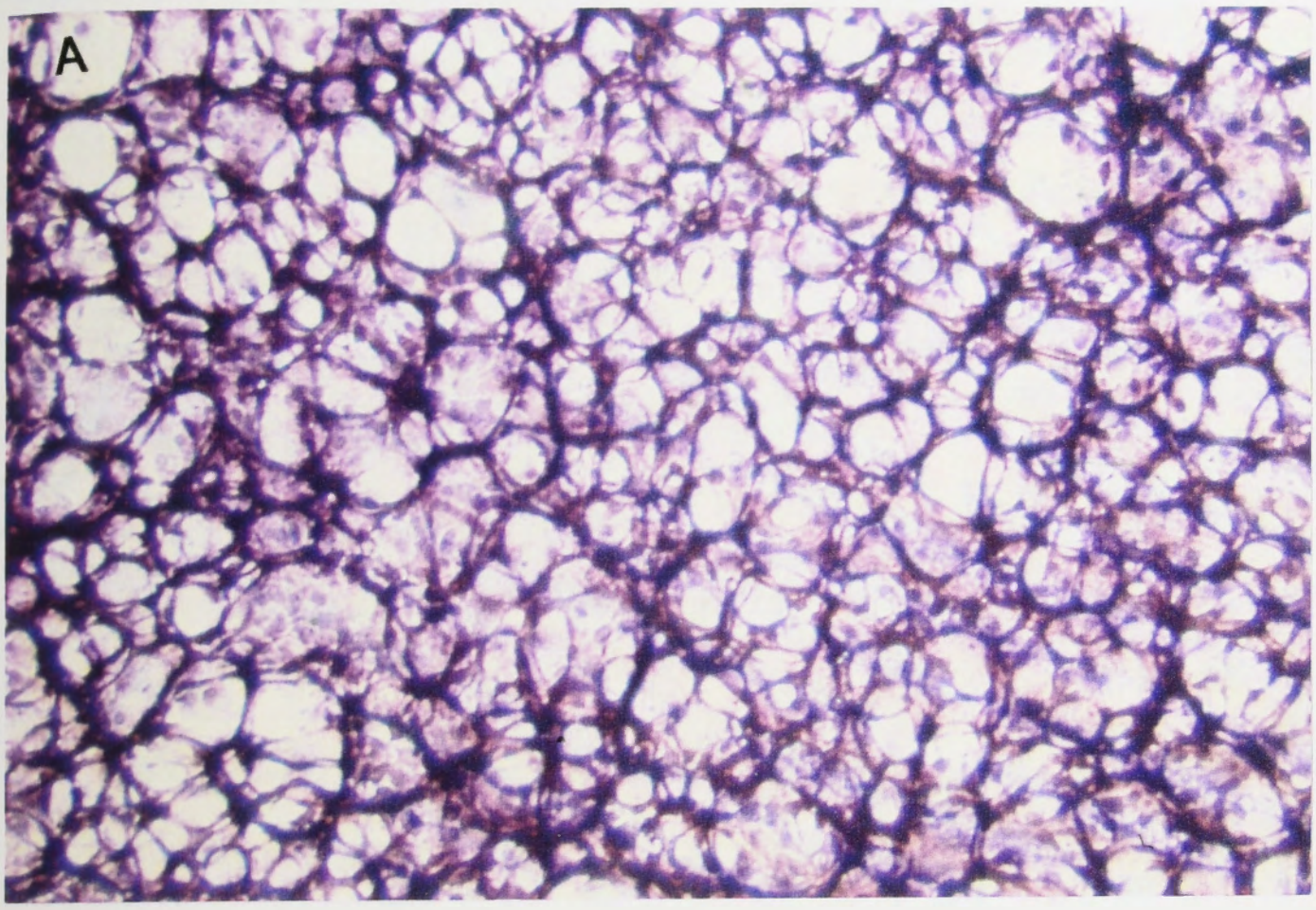
Figure 2. Microscopic appearance of reorganisation of thyrocyte follicle structures from dissociated cell suspensions.

(Fixation of these specimens was performed 5 days after initiation of the culture. Coverslips bearing the thyrocytes were all stained by means of H&E.)

(A) *Reorganisation of thyrocyte follicle structure of cryopreserved thyrocytes from foetal lambs.* Cryopreserved, thawed and dissociated thyrocytes from foetal lambs were reorganised into follicle-like or dome structures in culture medium containing 5%FCS and 20mU/ml TSH by establishing a high density of cells in a small area of culture surface. (Magnification ×195.)

(B) *Reorganisation of thyrocyte follicle structure of freshly isolated thyrocytes from neonatal DA rats.* Dissociated thyrocytes from neonatal DA rats were reorganised into follicle-like or dome structures in culture medium containing 5%FCS and 20mU/ml TSH by establishing a high density of cells in a small area of culture surface. (Magnification ×488).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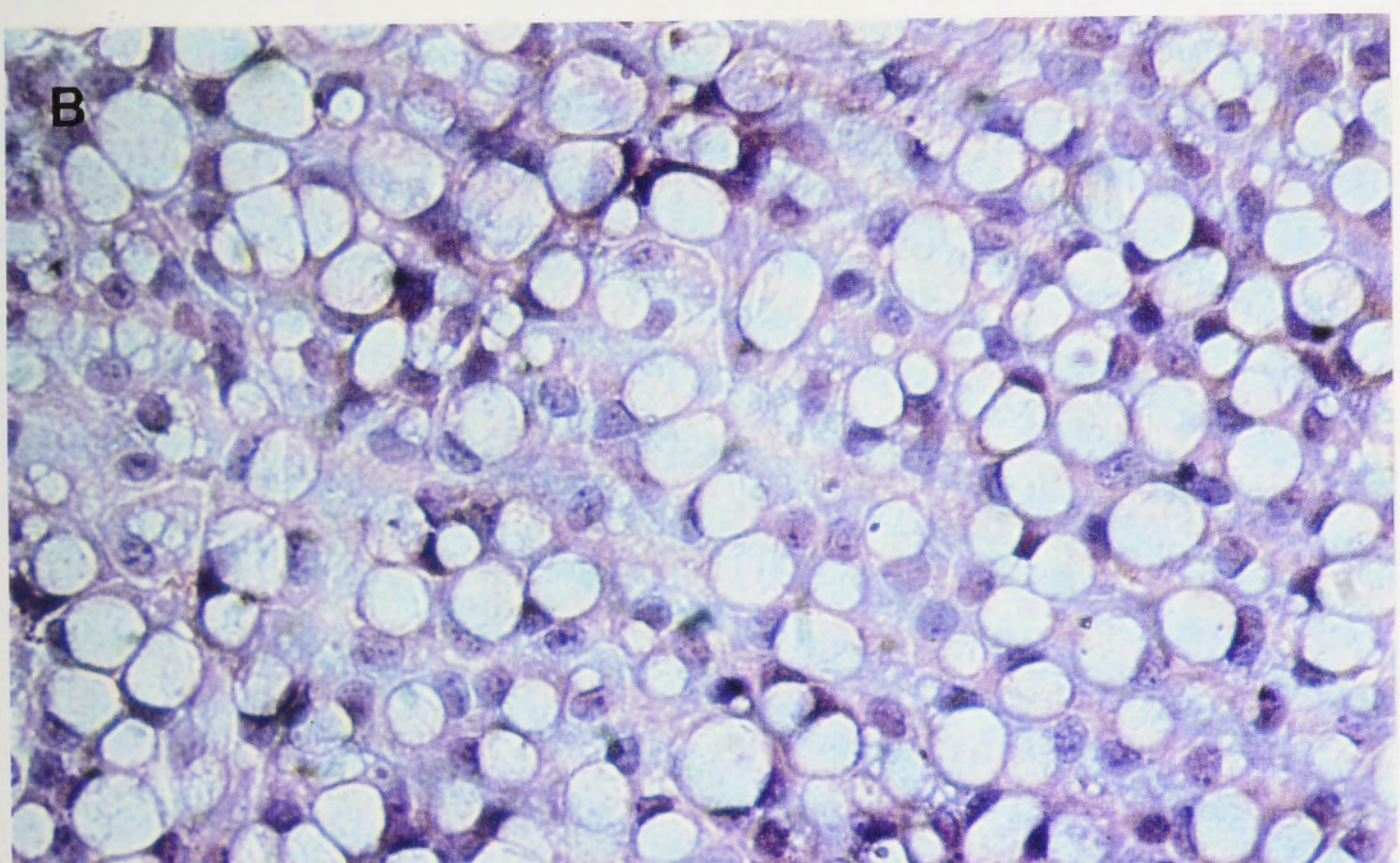
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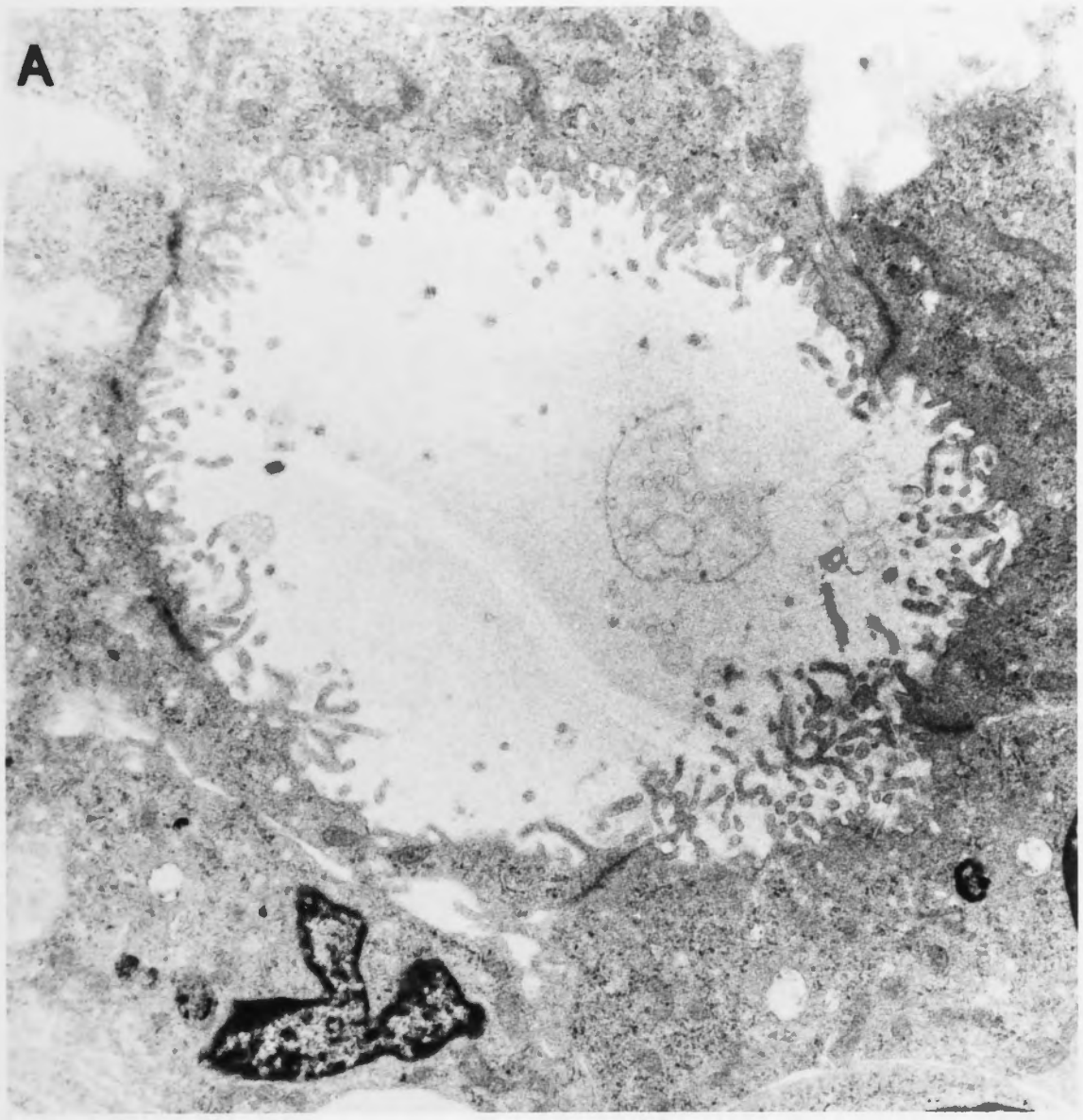
Figure 3. Electron microscopic appearance after reorganisation of thyrocyte follicle structures in culture.

(A) *Reorganisation of typical three-dimensional thyrocyte follicle structure by cryopreserved thyrocytes from foetal lambs.* (Magnification × 6400) Following cultivation for 5 days, these structures, characterised by the presence of a follicle lumen surrounded by thyroid epithelial cells with numerous microvillae at the apices of the cells, were formed.

(B) *Reorganisation of follicle structure by thyrocytes freshly isolated from neonatal DA rats.* (Magnification × 13,200) Similar features have been observed in cultivated thyrocytes from neonatal DA rats. Material was fixed 5 days after initiation of culture.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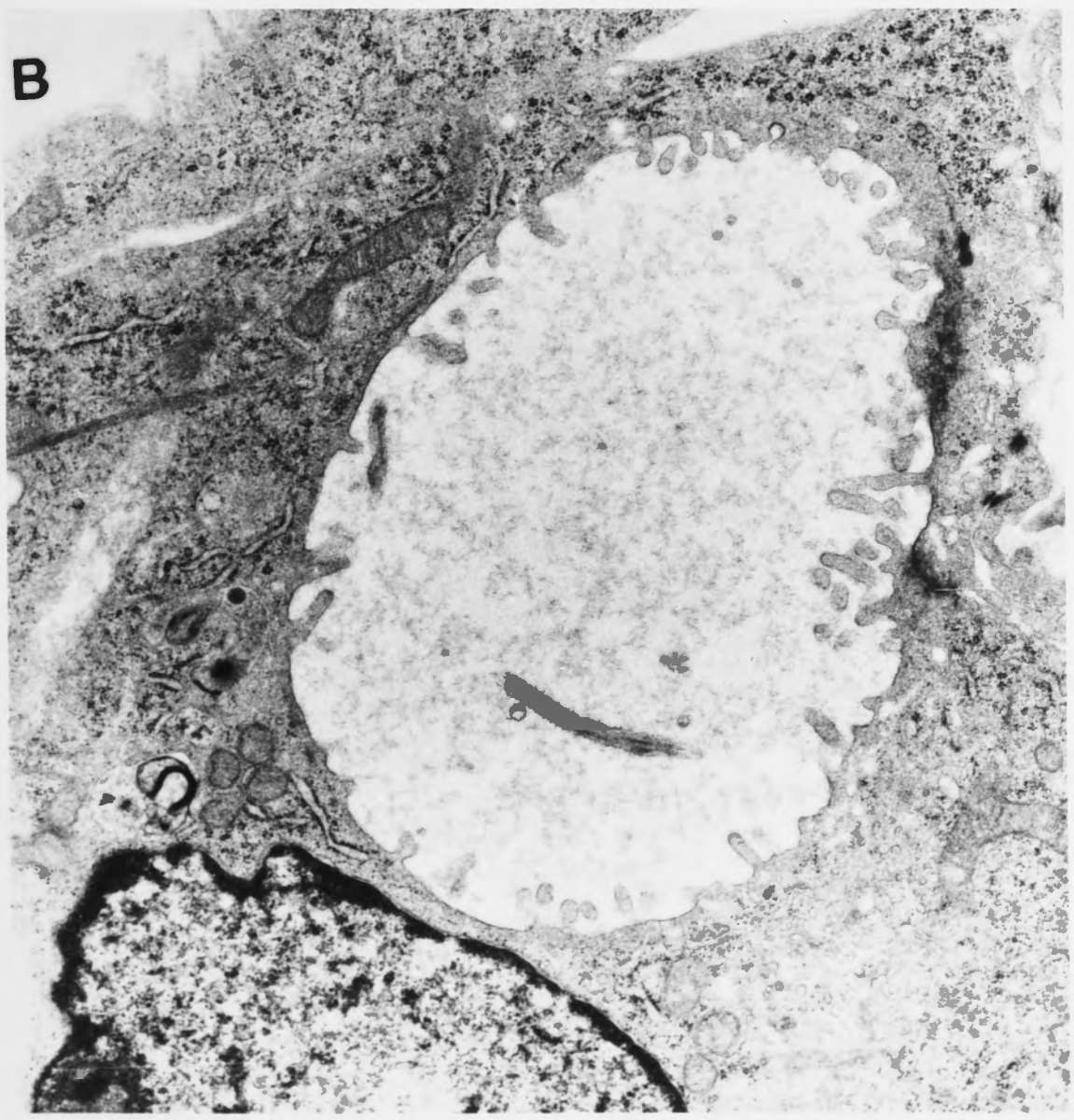
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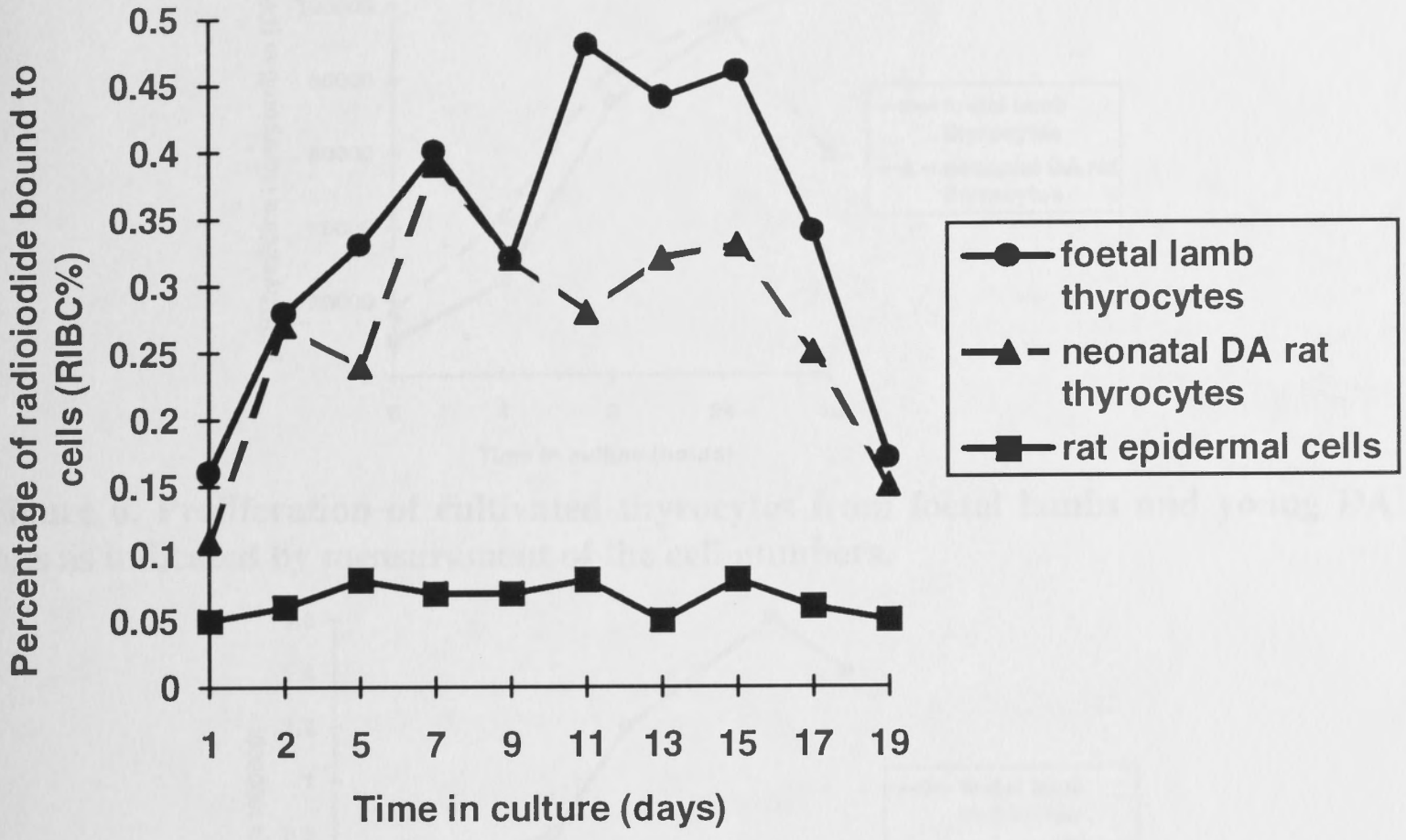


Figure 4. Iodide uptake by cultivated thyrocytes from foetal lambs and neonatal DA rats. In culture medium containing 5%FCS and 20mU/ml TSH, cryopreserved thyrocytes from foetal lambs or thyrocytes freshly isolated from neonatal DA rats exhibited mean value (±SE) of 0.36%±0.04% or 0.28%±0.03%, respectively, of radioiodide bound to cells (RIBC) during the first 15 days of cultivation. Iodide uptake by these cells was statistically significant ($P<0.01$) when compared with rat epidermal cells which bound a mean value (±SE) of 0.06%±0.01% of RIBC. The iodide uptake of foetal lamb or neonatal DA rat thyrocytes increased progressively until the fifteenth day of incubation. Each value for neonatal DA rat thyrocytes or for rat epidermal cells represented the mean value of triplicate samples of cells from a single source. Each value for foetal lamb thyrocytes was obtained from one test.

Figure 5.Proliferation of cultivated thyrocytes from foetal lambs or neonatal DA rats as indicated by [H³]thymidine incorporation into cellular DNA.

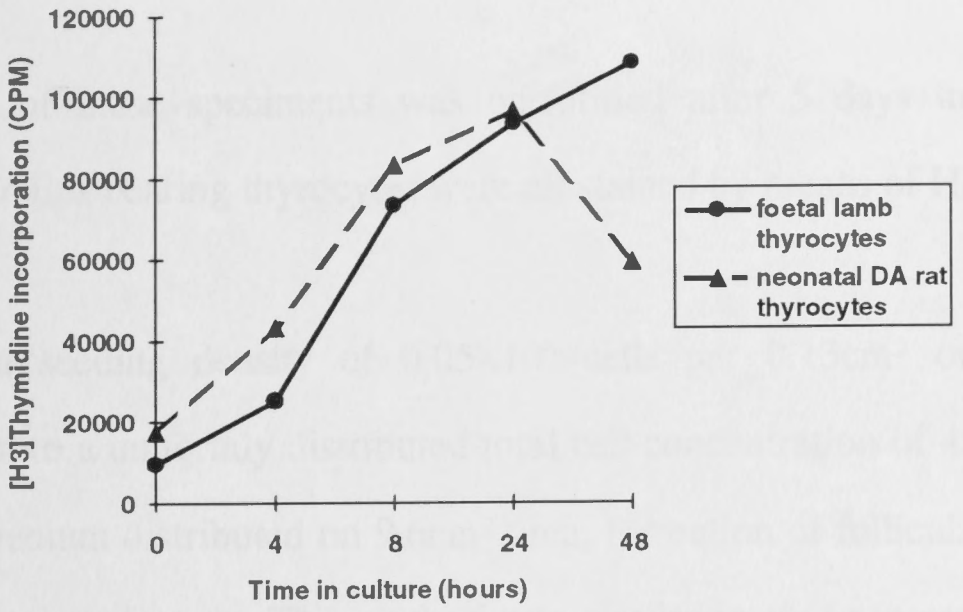


Figure 6. Proliferation of cultivated thyrocytes from foetal lambs and young DA rats as indicated by measurement of the cell numbers.

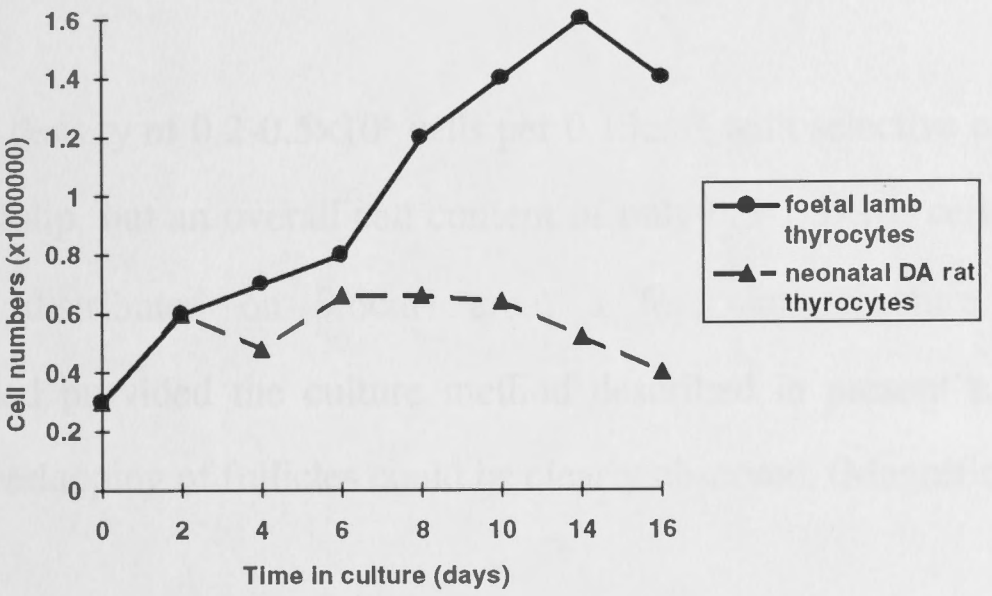


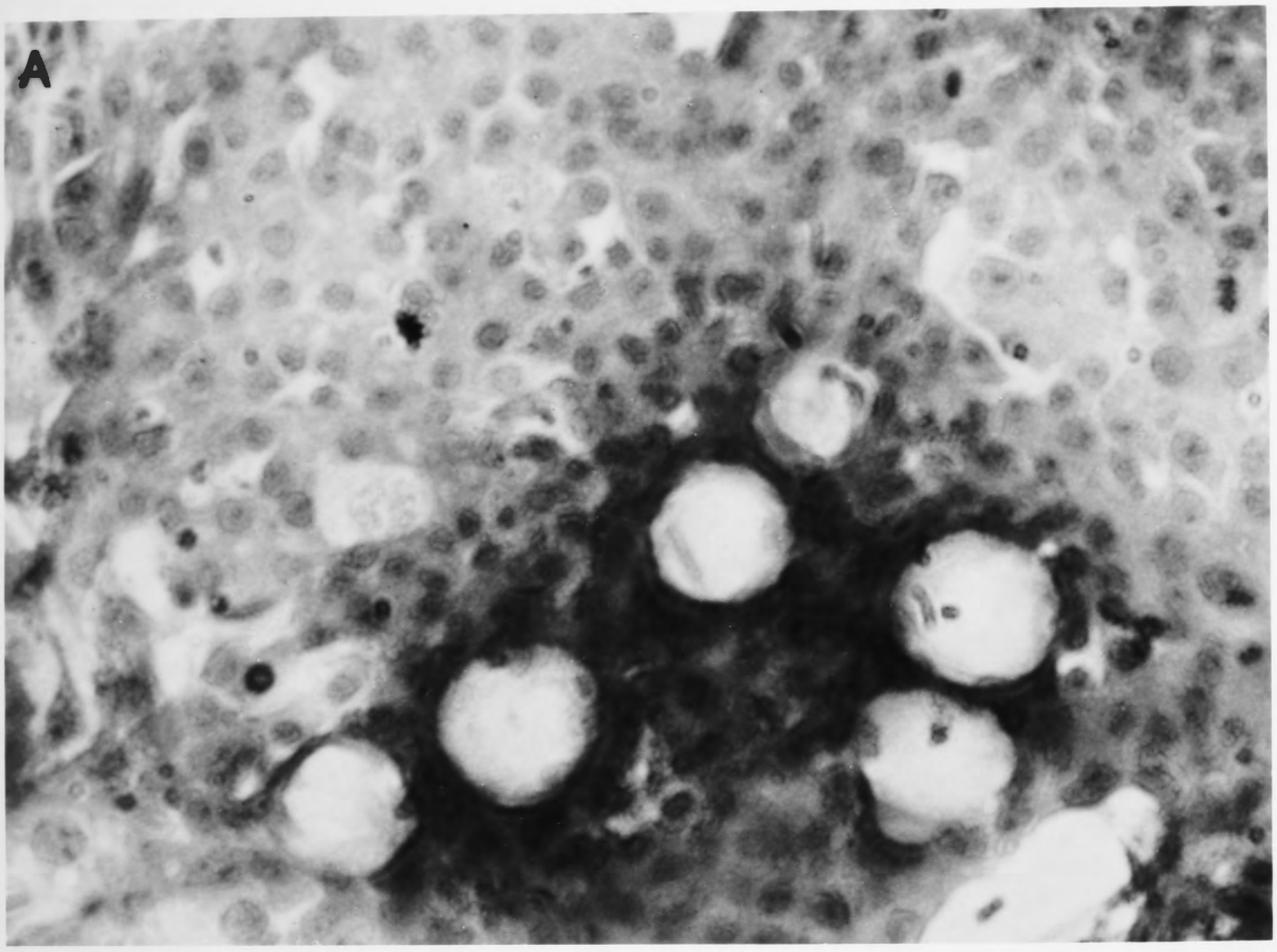
Figure 5-6. In culture medium containing 5%FCS and 20mU/ml TSH, cryopreserved thyrocytes from foetal lambs and freshly isolated thyrocytes from young DA rats have a doubling time of 4 hours (Figure 5) and a log phase of 2 days (Figure 5-6). The growth of rat thyrocytes then entered the plateau phase whereas foetal lamb thyrocytes continued to proliferate moderately after the second day of incubation (Figure 6). Each value obtained using rat cells represented the mean value of triplicate samples. The foetal lamb thyrocyte points each represented a single reading.

Figure 7. The influence of cell density on the morphology of cultivated thyrocytes.

(Fixation of these speciments was performed after 5 days incubation of the thyrocytes. Coverslips bearing thyrocytes were all stained by means of H&E stain.)

(A) At a seeding density of 0.05×10^6 cells per $0.13cm^2$ on the coverslip, equivalent to a uniformly distributed total cell concentration of 4×10^6 cells in 3ml culture medium distributed on $9.6cm^2$ area, formation of follicular structures was not a prominnt feature. This method was similar to that reported by Yap *et al.* (1987). (Magnification×488)

(B) At a density of $0.2\text{-}0.5\times10^6$ cells per $0.13cm^2$ with selective concentration on the coverslip, but an overall cell content of only $0.5\text{-}1.5\times10^6$ cells in 3ml culture medium distributed on $9.6cm^2$ area, a follicular structure was regularly established provided the culture method described in present experiments was used. Overlapping of follicles could be clearly observed. (Magnification×195)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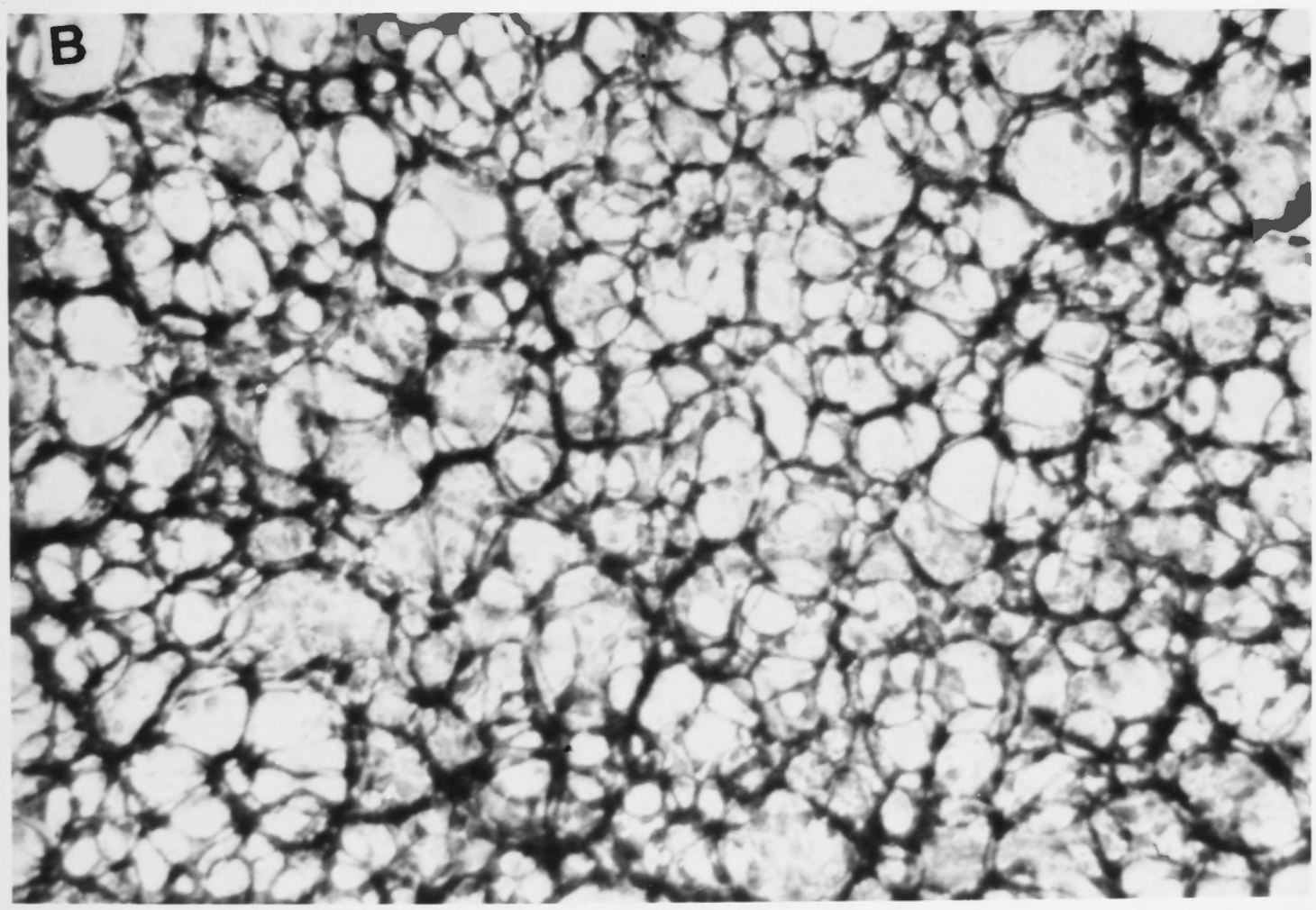
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Table 1. Comparison of cultivated cell density and total cell concentration utilised in the present experiments and those of Yap *et al.* (1986).

	Culture method of Yap *et al.*	Present culture method
Cultivated cell density (per 0.13cm^2)	0.05×10^6	$0.2\text{-}0.5\times10^6$
Total cell concentration (in 3ml culture medium on 9.6cm^2 area)	$3\text{-}4\times10^6$	$0.5\text{-}1.5\times10^6$

In comparison with the culture method of Yap *et al.*, cultivated thyrocytes in the present experiments were placed onto coverslips at a high cell density ($0.2\text{-}0.5\times10^6$ in 0.13cm^2 area), whereas the overall cell concentration (0.5×10^6 in 3ml culture medium on 9.6cm^2 area) was lower than that of Yap *et al.*

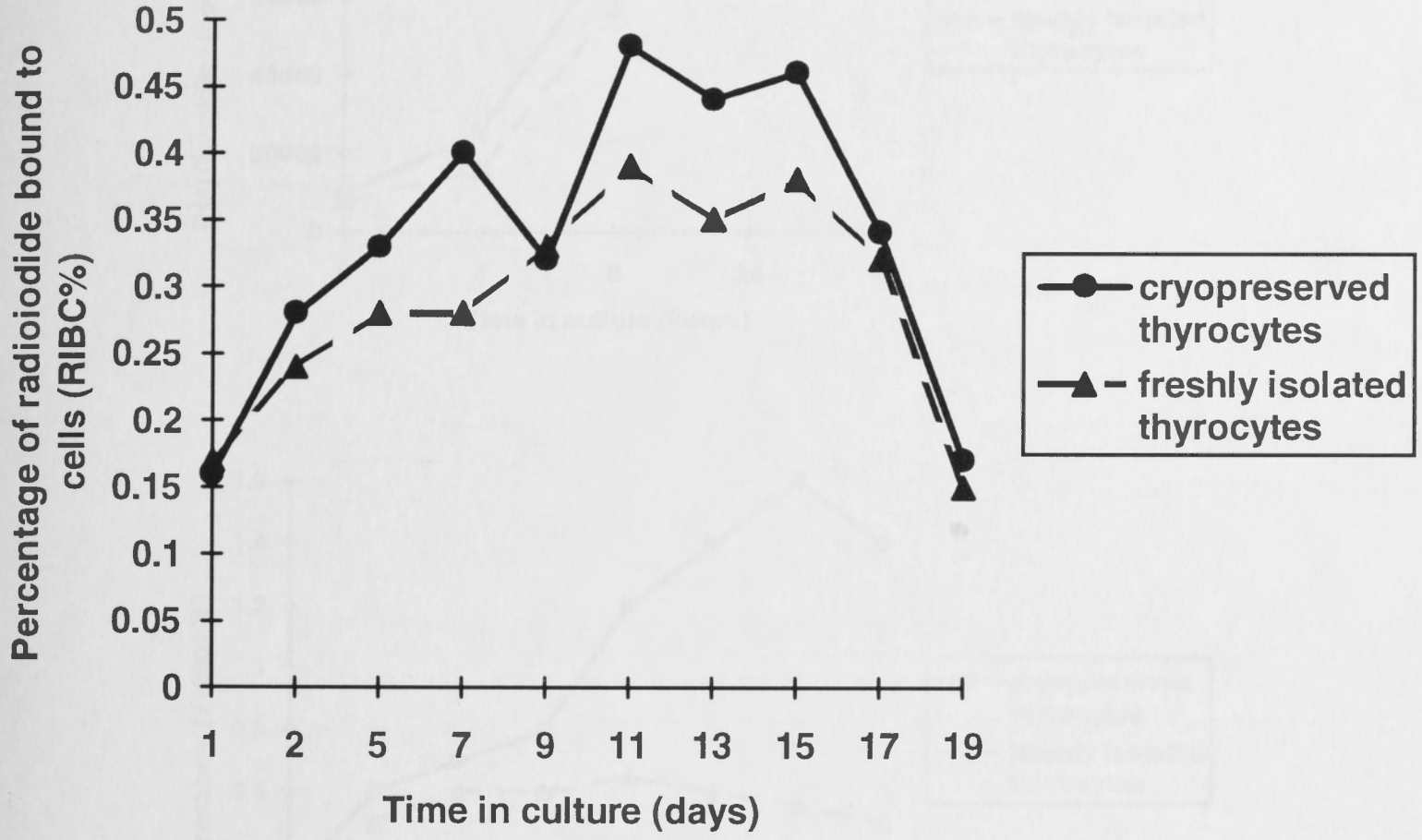
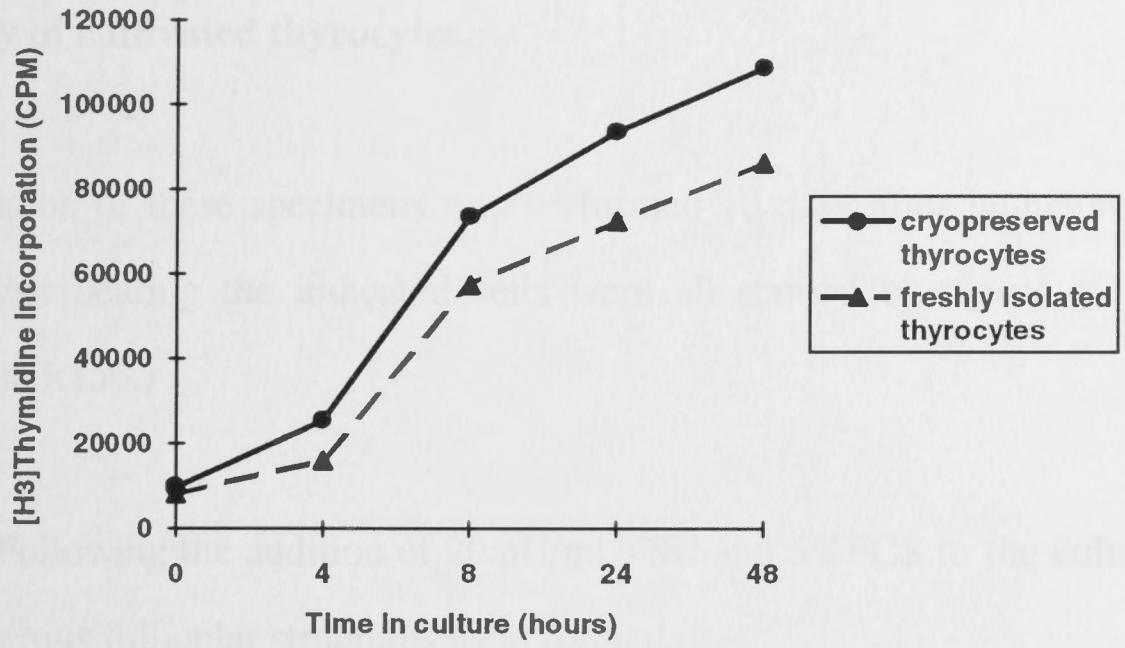


Figure 8. Comparison of iodide uptake by cryopreserved and freshly isolated foetal lamb thyrocytes. In the case of foetal lamb thyrocytes, there was no significant difference between the capacity for iodide uptake of cryopreserved and freshly isolated thyrocytes. Cryopreserved thyrocytes had a mean value (±SE) of 0.36%±0.04% RIBC, while freshly isolated thyrocytes had a mean value of 0.30%±0.03% RIBC. Each value represented the result of one test. All the thyrocytes examined were derived from a single batch.



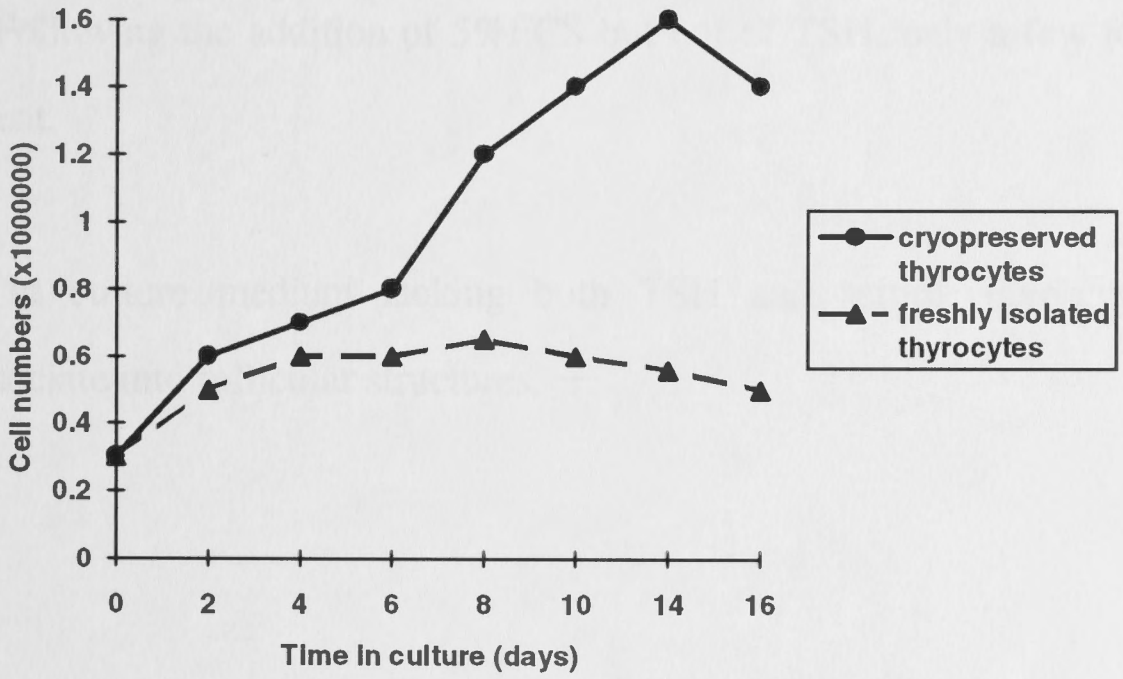


Figure 9-10. Comparison of the proliferation of cryopreserved and freshly isolated foetal lamb thyrocytes. Both cryopreserved and freshly isolated foetal lamb thyrocytes have a doubling time of 4 hours. After 2 days in the log phase, freshly isolated thyrocytes no longer proliferated whereas cryopreserved cells continued to do so. Each value in Figure 9 represented the mean value of triplicate samples. Each value in Figure 10 was obtained from one test. All thyrocytes examined were derived from the same lamb.

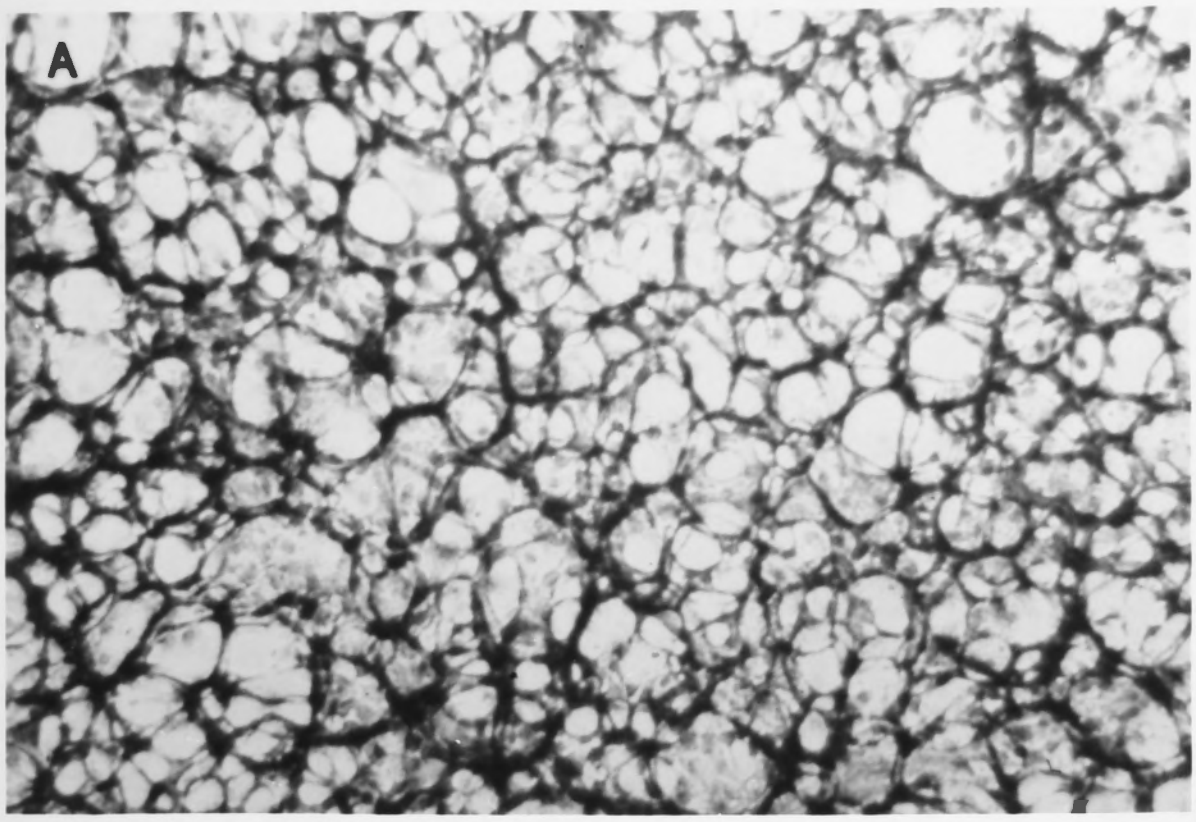
Figure 11. Influence of the concentration of TSH in the culture medium on the morphology of cultivated thyrocytes.

(Fixation of these specimens was performed 10 days after initiation of cultures. The coverslips bearing the indicated cells were all stained by means of H&E stain. Magnification ×156.)

(A) Following the addition of 20mU/ml TSH and 5%FCS to the culture medium, numerous follicular structures were formed.

(B) Following the addition of 5%FCS but not of TSH, only a few follicles were evident.

(C) In culture medium lacking both TSH and serum, thyrocytes did not reassociate into follicular structures.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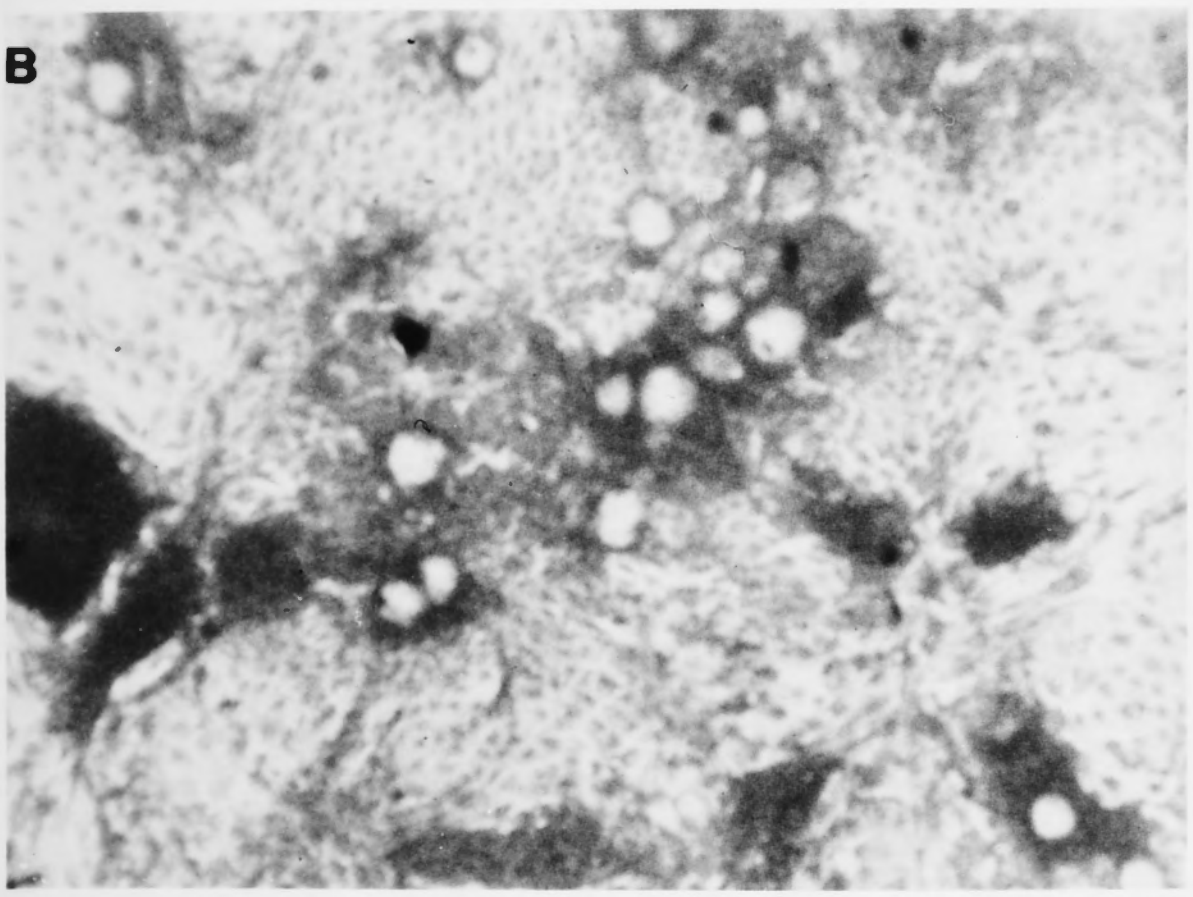
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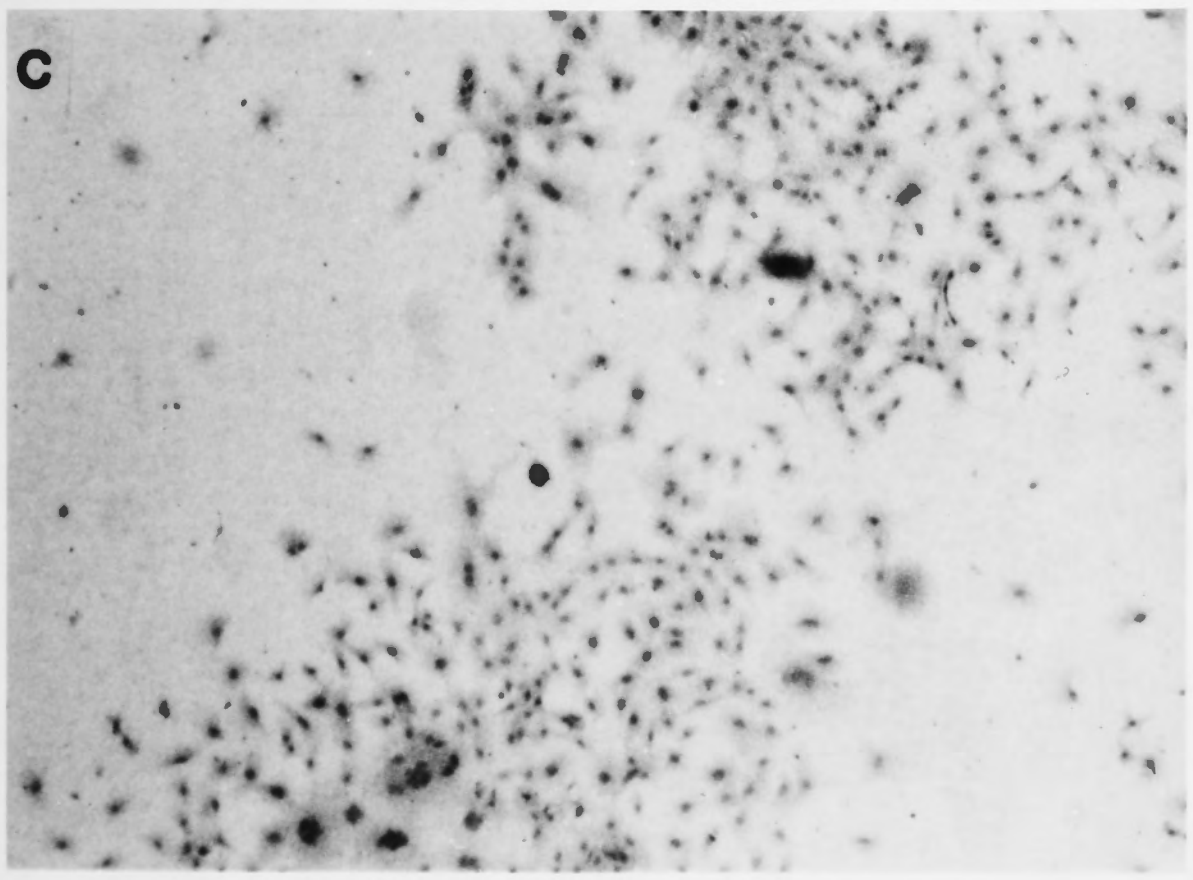
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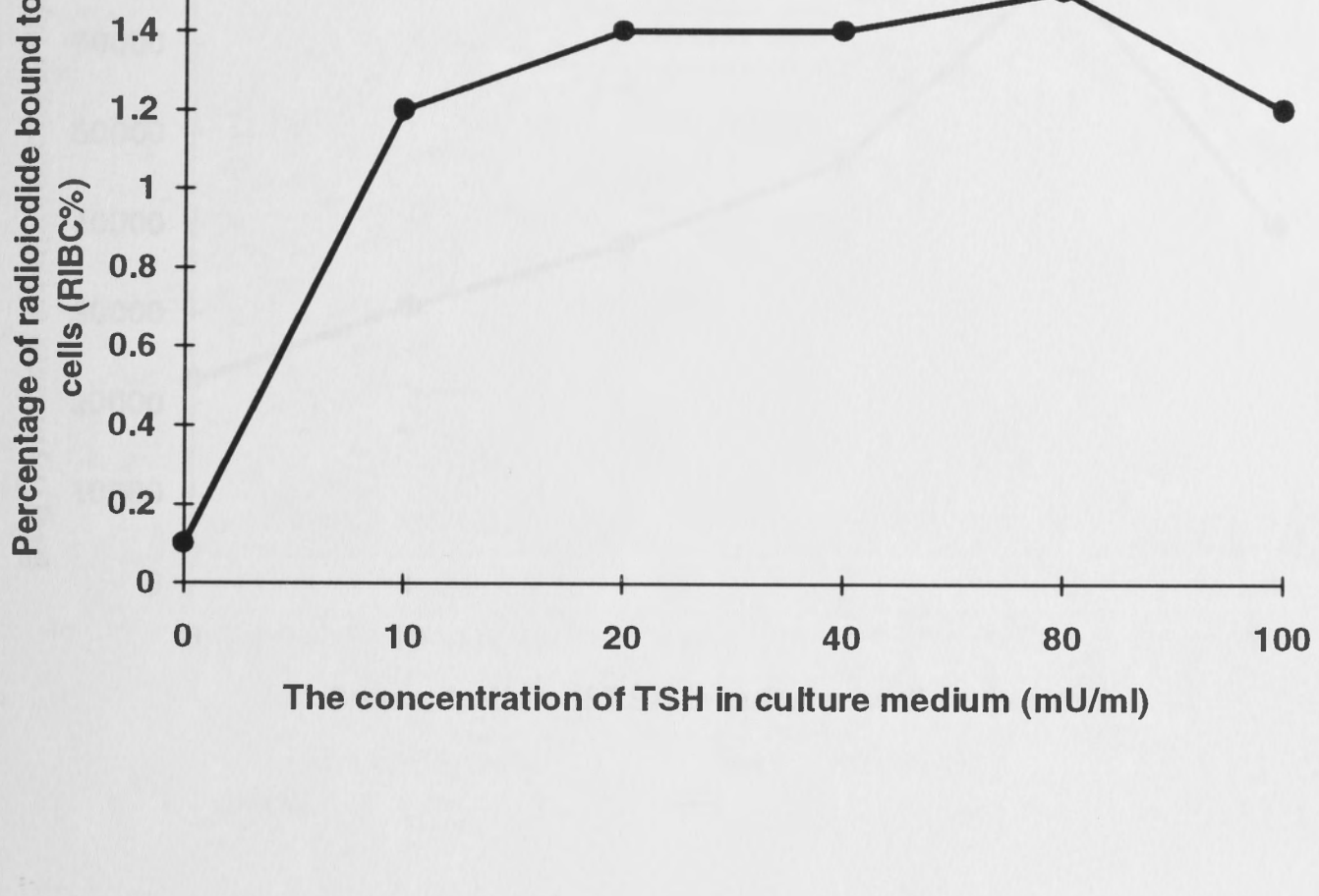


Figure 12. The influence of TSH concentration in culture medium on iodide uptake by neonatal DA rat thyrocytes. Iodide uptake by rat thyrocytes was tested after 2 days of initiating culture of the thyrocytes. Compared with the outcome in the absence of TSH from the culture medium (RIBC = 0.1%), thyrocytes in culture medium containing TSH (10mU/ml) manifested a considerably higher iodide uptake (1.2%RIBC). However, the iodide uptake of thyrocytes did not continue to rise with TSH concentration in the culture medium after this reached 20mU/ml. Each value in Figure 12 represented the mean value of triplicate samples. All samples were from the same batch of thyrocytes.

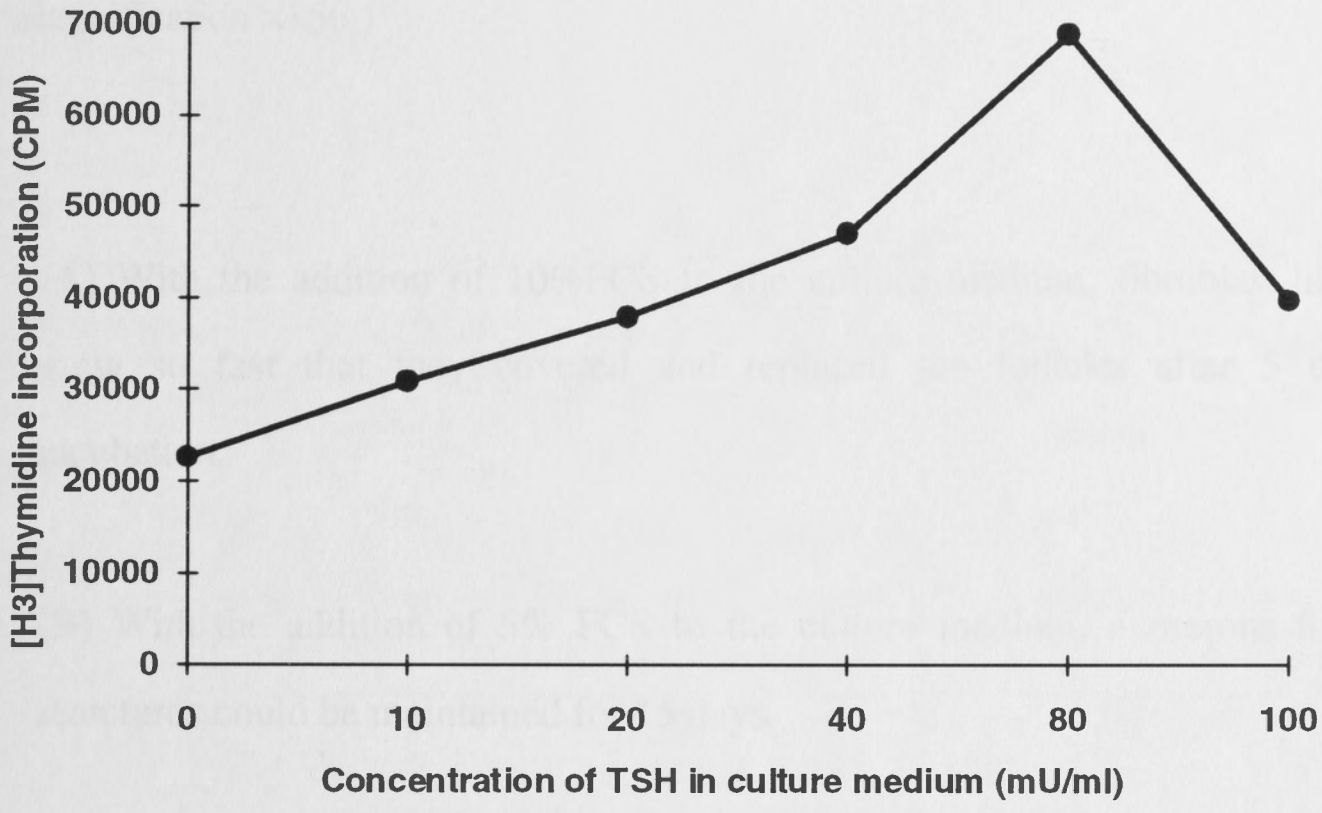


Figure 13. The influence of the concentration of TSH in culture medium on the proliferation of thyrocytes from neonatal DA rats. [H^3]Thymidine incorporation into rat thyrocytes was tested 24 hours after initiating culture of the thyrocytes. The values of [H^3]thymidine incorporation rise together with increase in the concentration of TSH in the culture medium. The presence of 80mU/ml TSH in the culture medium was associated with maximum thyrocyte proliferation. Each value in Figure 13 represented the mean value of triplicate samples. All samples were from a single batch of thyrocytes.

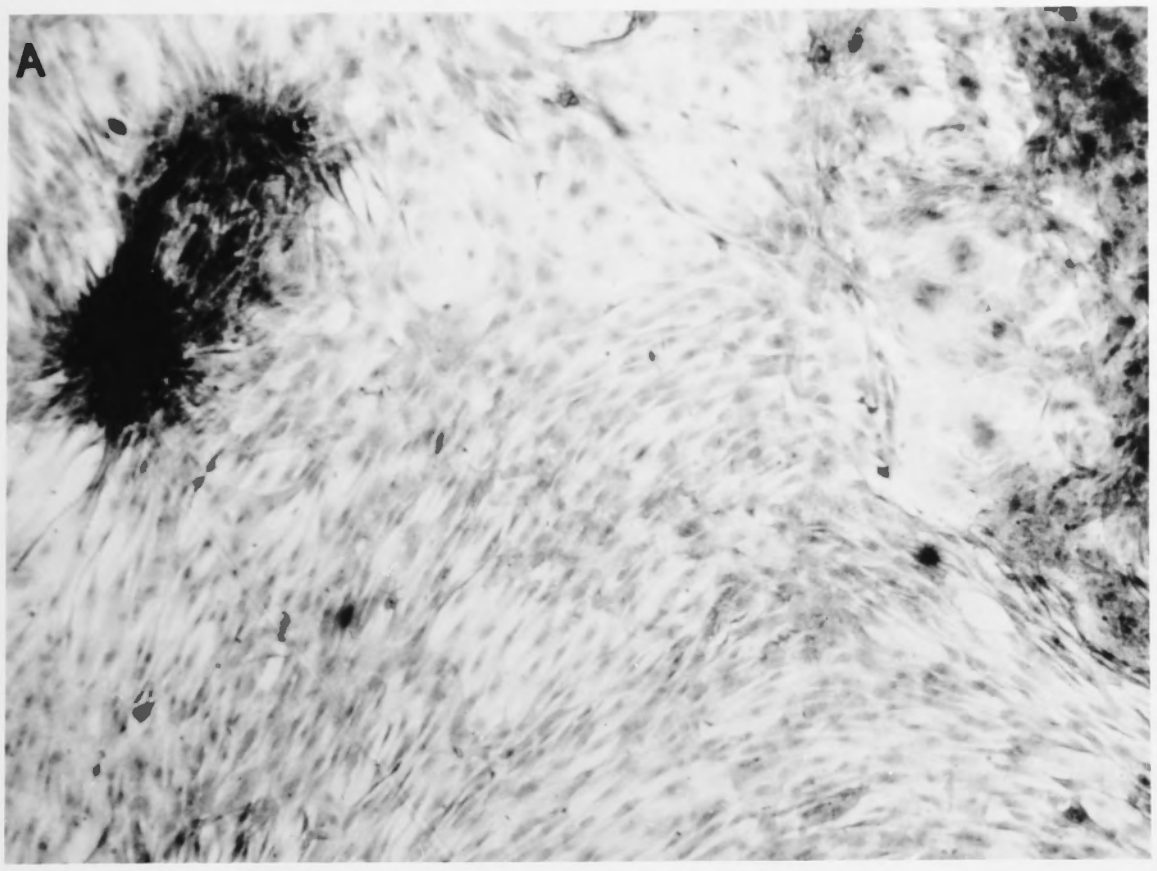
Figure 14. The influence of serum concentration in the culture medium on morphology of cultivated thyrocytes.

(The coverslips bearing the indicated cells were all stained by means of H&E stain. Magnification ×156.)

(A) With the addition of 10%FCS to the culture medium, fibroblast-like cells grew so fast that they covered and replaced the follicles after 5 days of incubation.

(B) With the addition of 5% FCS to the culture medium, numerous follicular structures could be maintained for 15 days.

(C) With the addition of only 2.5%FCS to the culture medium, thyrocytes grew so slowly that only a few follicles had appeared by 10 days of culture.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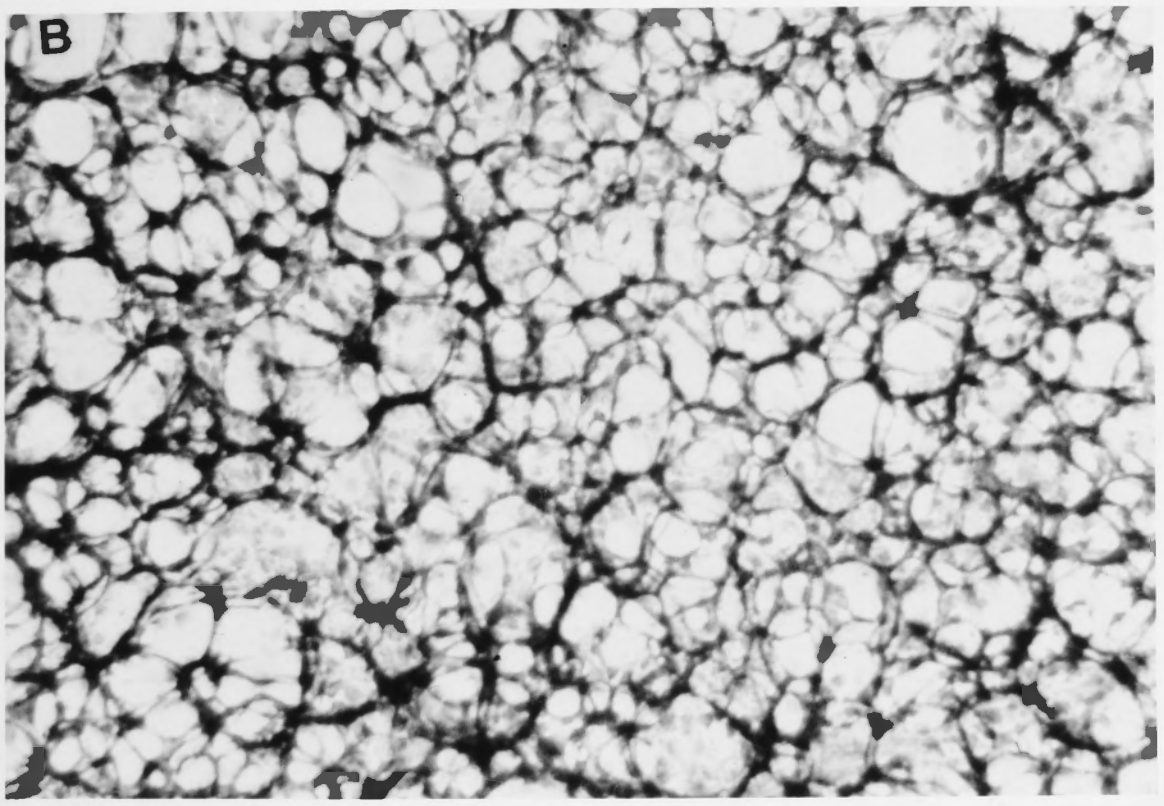
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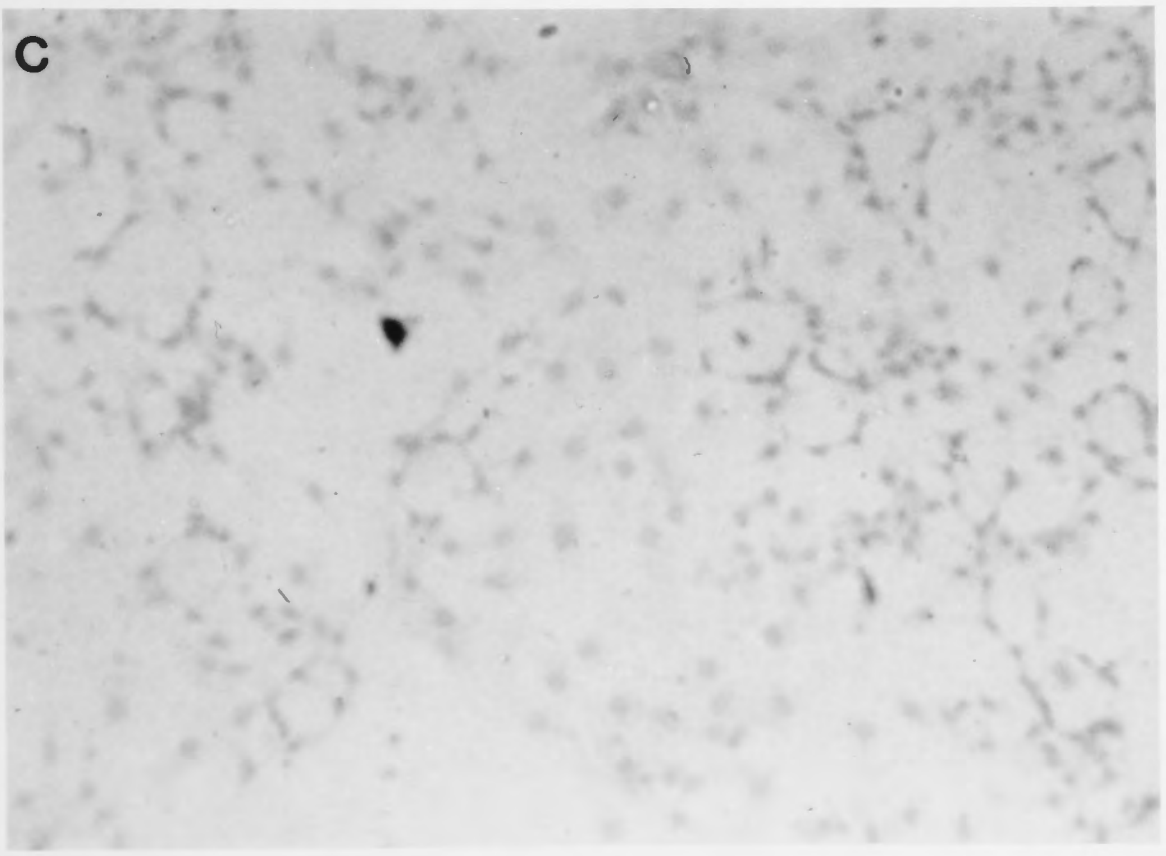
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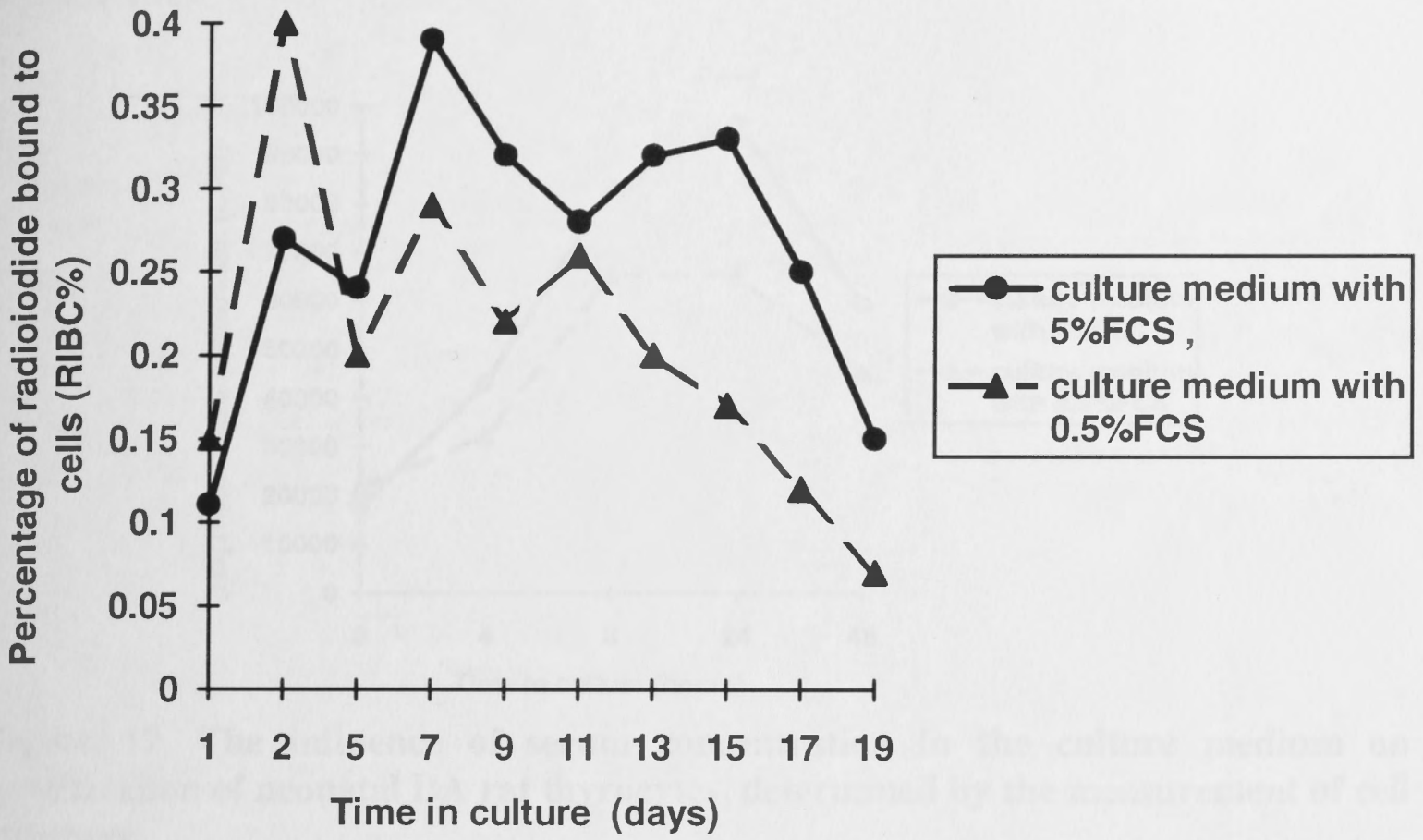


Figure 15. The influence of serum concentration in culture medium on iodide uptake by neonatal DA rat thyrocytes. Within 15 days of initiating cultivation, thyrocytes in medium with low serum concentrations showed a slightly lower capacity for iodide uptake (mean values of 0.23%±0.03%RIBC) in comparison with cells in medium containing 5%FCS which had had a mean value of 0.28%±0.03%RIBC. Additionally, the decline of iodide uptake by thyrocytes in culture medium with 0.5%FCS started at the eleventh day of incubation. This was earlier than the time at which this happened (fifteenth day of incubation) for thyrocytes in culture medium with 5%FCS. Each value in Figure 15 represented the mean value of triplicate samples. All samples in each item were from the same batch of thyrocytes.

Figure 16. The influence of serum concentration on proliferation of cultivated neonatal DA rat thyrocytes, determined by [H³]thymidine incorporation into cellular DNA.

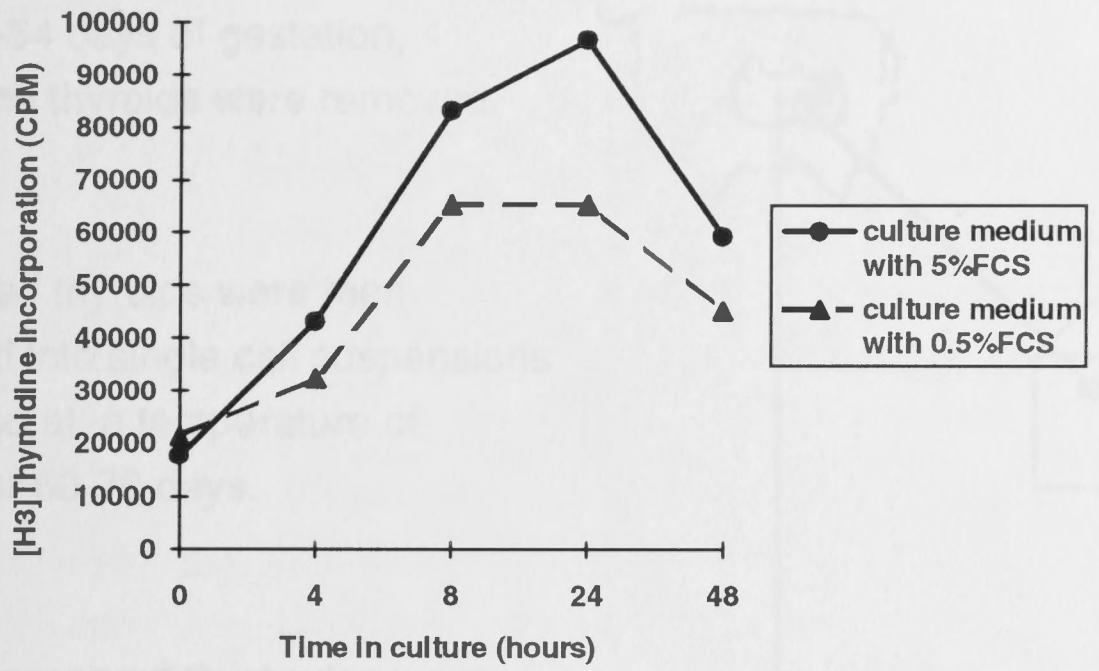


Figure 17. The influence of serum concentration in the culture medium on proliferation of neonatal DA rat thyrocytes, determined by the measurement of cell numbers.

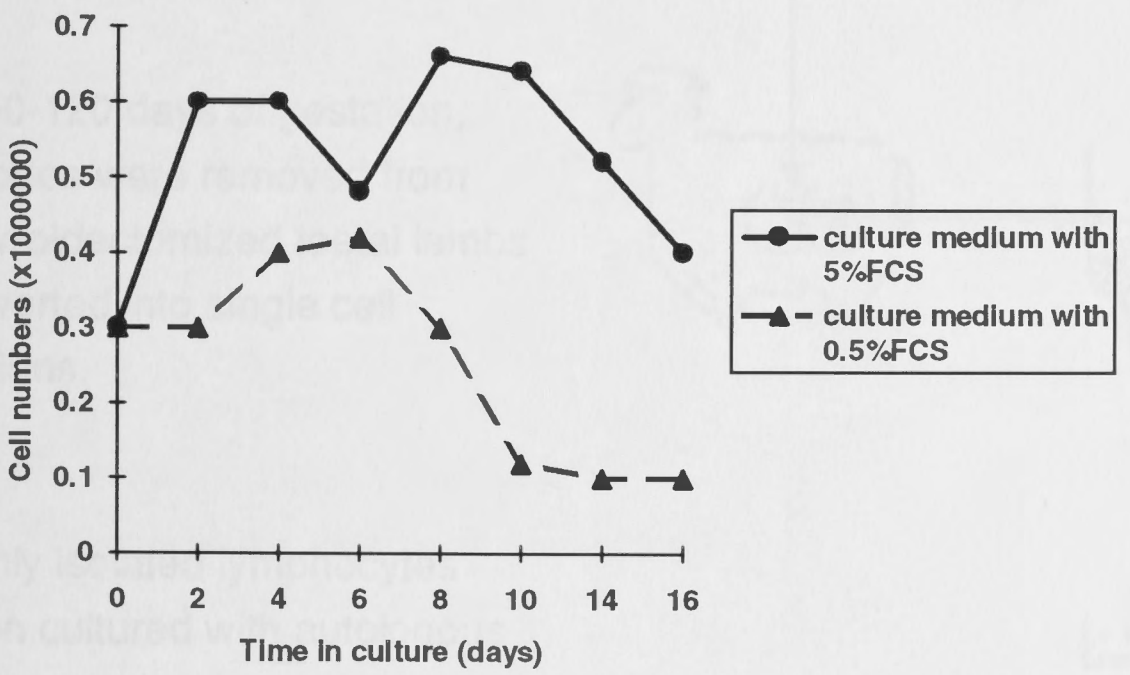


Figure 16-17. In culture medium with 0.5%FCS, rat thyrocytes underwent little proliferation and cell numbers began to decline at an earlier time (by the eighth day). Each value in Figure 16-17 represented the mean value of triplicate samples. All samples were from the same batch of thyrocytes.

Figure 18. Experimental plan 1.

(1) At 51-54 days of gestation, foetal lamb thyroids were removed.

(2) Excised thyroids were then converted into single cell suspensions and stored at a temperature of -196°C for 60-70 days.

(3) Cryopreserved thyrocytes were cultured in the presence of TSH for 3 days before incubation with autologous lymphocytes.

(4) At 100-120 days of gestation, lymph nodes were removed from these thyroidectomized foetal lambs and converted into single cell suspensions.

(5) Freshly isolated lymphocytes were then cultured with autologous thyrocytes that had been cultured for 3 days previously.

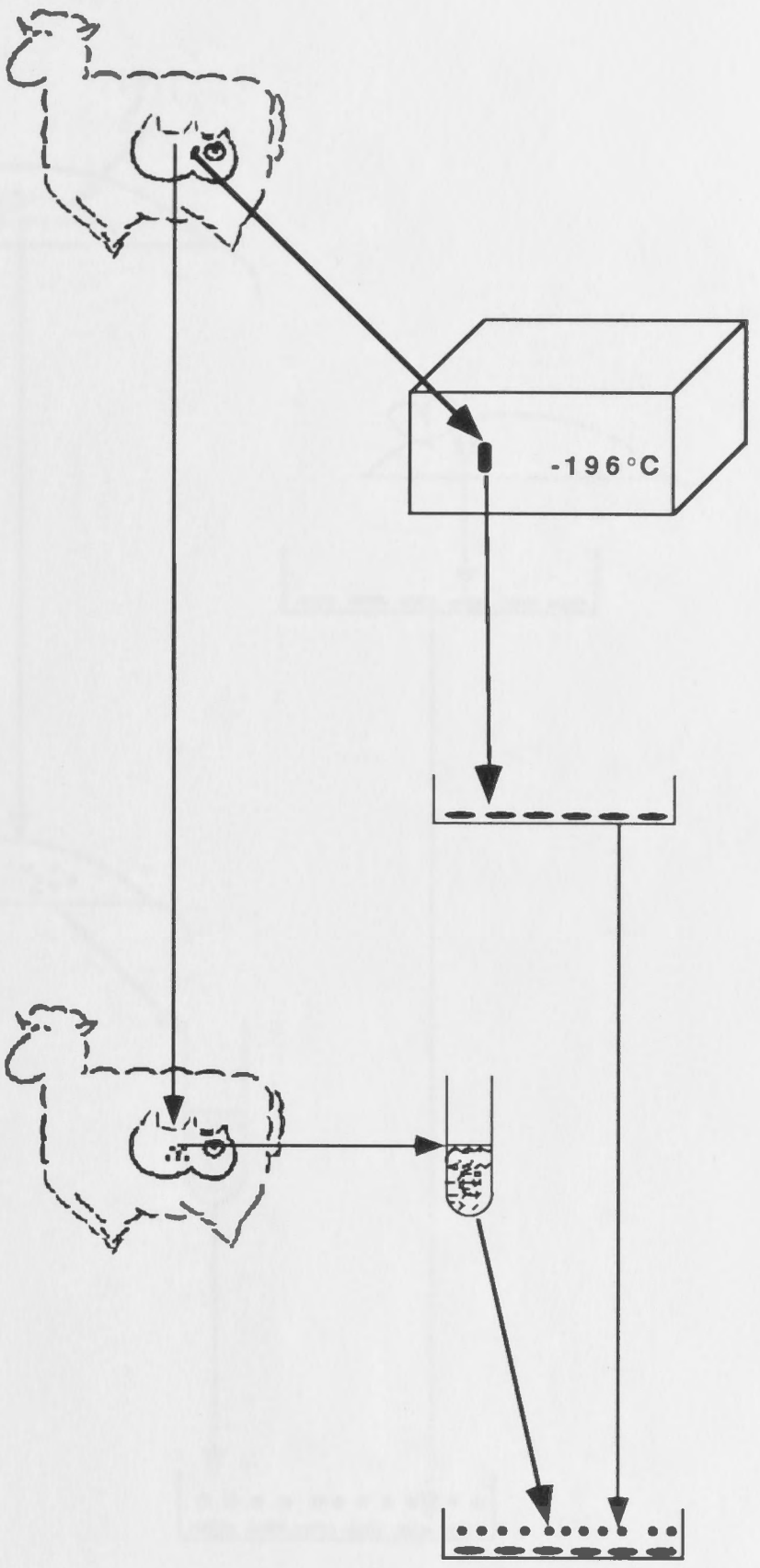


Figure 19. Experimental plan 2.

(1) At 17 days gestation, foetal DA rats received a dose of ^{131}I sufficient to disrupt further development of the thyroid gland.

(2) Thyroid glands from syngeneic neonatal DA rats were removed and converted into single cell suspensions which were then cultured for 5 days before incubation with lymphocytes from ^{131}I exposed rats.

(3) Lymph nodes were removed from 1 year old ^{131}I exposed DA rats and converted into single cell suspensions.

(4) Freshly isolated lymphocytes were then incubated with syngeneic thyrocytes that had been previously cultured for 5 days.

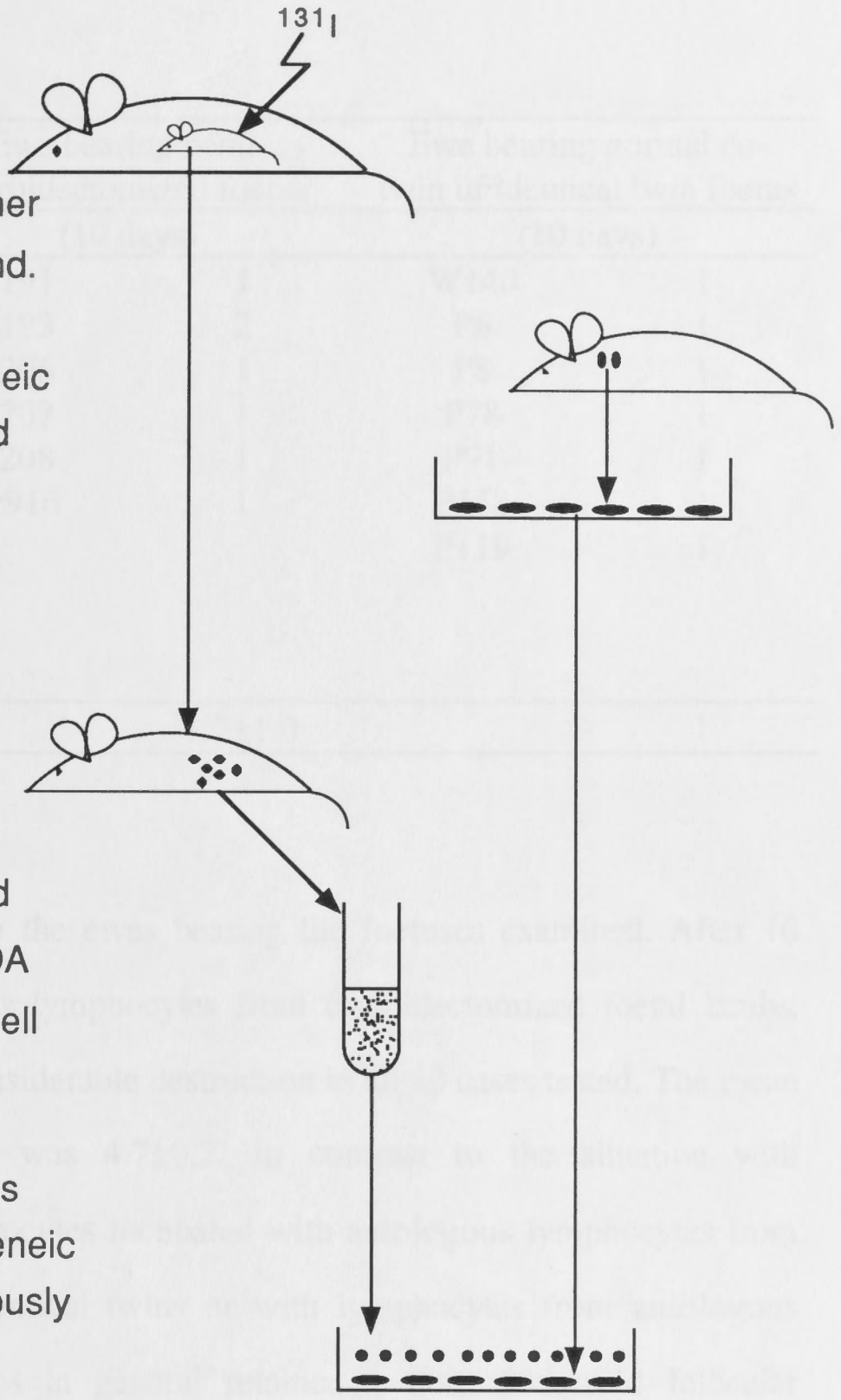


Table 2. Comparison of the grade of autologous thyrocyte damage achieved in tissue culture by lymphocytes from thyroidectomized, hemi-thyroidectomized and normal foetal lambs.

Ewe bearing thyroidectomized foetus (10 days)		Ewe bearing hemi-thyroidectomized foetus (10 days)		Ewe bearing normal co-twin of identical twin foetus (10 days)	
Or30	4	Y191	1	W140	1
Or230	5	Y193	2	P6	1
Or235	5	Y206	1	P8	1
Or554	5	Y207	1	P78	1
W123	5	Y208	1	P91	1
W195	4	Or916	1	P118	1
W196	5			P119	1
W199	4				
W208	5				
P30	5				
mean±SE	4.7±0.2		1.2±1.0		1

The identification numbers refer to the ewes bearing the foetuses examined. After 10 days of incubation with autologous lymphocytes from thyroidectomized foetal lambs, cultivated thyrocytes underwent considerable destruction in all 10 cases tested. The mean grade of damage in these cases was 4.7±0.2. In contrast to the situation with thyroidectomized foetal lambs, thyrocytes incubated with autologous lymphocytes from the normal co-twin of pairs of identical twins or with lymphocytes from autologous hemi-thyroidectomized foetal lambs in general retained a normal thyroid follicular structure with only a slight degree (grade 1 or 1.2±1.0 respectively) of destruction in a few areas of thyrocyte monolayers. The mean grade of 4.7 for the group of thyroidectomized foetuses was significantly higher than the mean grades for the 2 other groups ($P<0.01$).

Table 3. Grade of damage of thyrocytes after incubation with autologous lymphocytes from partially thyroidectomized foetal lambs.

Ewe bearing partially thyroidectomized foetus	Grade of thyrocyte damage (10 days)
Or1001	1
W44	2
W63	1
W164	1
W185	1
W203	1
W215	1
W244	4
P91	4
mean±SE	1.8±0.4

In 9 cases, bilateral thyroidectomy was undertaken at 51-54 days of gestation, but was found, at subsequent post mortem, to have been incomplete. 7 out of 9 cases retained normal follicle structure without major damage, but 2 cases exhibited considerable degeneration and necrosis of thyrocytes after incubation with autologous lymphocytes for 10 days.

Table 4. Comparison of the grade of thyrocyte damage by lymphocytes from ^{131}I exposed and normal DA rats.

^{131}I exposed DA rats	Grade of thyrocyte damage (10 days)	Normal DA rats	Grade of thyrocyte damage (10 days)
1/1	2	1	1
2/1	2	2	2
3/1	2	3	2
4/1	5	4	1
1/5	5	5	1
2/5	5	6	1
1/6	2	7	2
1/7	2	8	1
2/7	3	9	1
3/7	3	10	1
4/7	3	11	1
1/4	3	12	2
2/4	3	13	1
1/9	3	14	1
2/9	3	15	2
1/11	4	16	1
2/11	3	17	1
1/12	3	18	1
2/12	2	19	1
1/10	3	20	1
1/13	4	21	1
2/13	4	22	1
3/13	4	23	1

In 17 out of 23 (74%) ^{131}I exposed DA rats, cultivated syngeneic thyrocytes were damaged (>grade 2) after incubation with lymphocytes from these rats. The thyrocytes in 10 out of these 17 cases showed only a moderate degree of destruction(<grade 4) with degeneration or necrosis of thyrocytes occurring only in infrequent foci. In the other 7 cases, severe destruction was observed (>grade 4). Thyrocytes that had been incubated with normal, syngeneic lymphocytes maintained their normal follicular structure with only occasional foci of slight destruction.

Figure 20. Microscopic appearance of cytotoxic responses against DA rat thyrocytes.

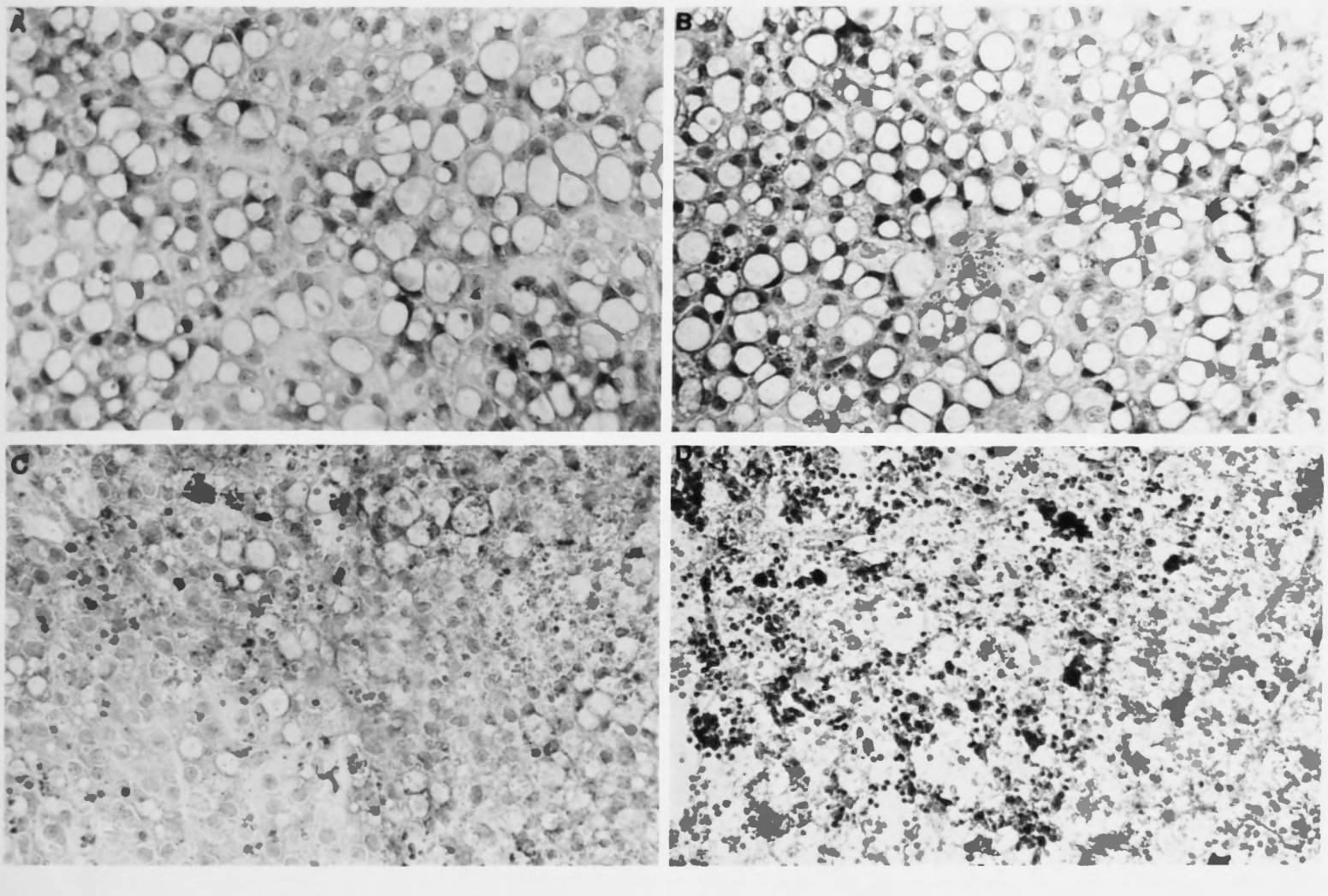
(Fixation of these specimens was performed 10 days after addition of lymphocytes to thyrocyte monolayers in the case of B, C and D. The coverslips bearing the indicated cells were all stained by means of H&E stain. Magnification ×244.)

(A) *Cultivated DA rat thyrocytes alone.* Intact thyrocyte follicles were retained.

(B) *Cultivated thyrocytes incubated with lymphocytes from syngeneic normal DA rats.* Intact thyrocyte follicles were retained in most parts of cultivated autologous thyrocyte monolayers.

(C) *Cultivated thyrocytes incubated with syngeneic lymphocytes from* ^{131}I *exposed rats.* The thyrocyte follicles have sustained severe damage. Major destruction was apparent at the apices of those epithelial thyrocytes which had organised into thyroid follicles. The interconnection of follicles was almost completely lost, to be replaced by single, degenerating follicles.

(D) *Cultivated thyrocytes incubated with syngeneic lymphocytes from* ^{131}I *exposed rats.* In the most severe cases of which this is an example, complete lysis of thyrocytes has been observed.


A
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D

Table 5. Comparison of [H^3] thymidine incorporation by lymphocytes from normal and ^{131}I exposed DA rats after different periods of incubation with syngeneic thyrocytes.

Normal rat	[H^3] thymidine incorporation (CPM)			^{131}I exposed rats	[H^3] thymidine incorporation (CPM)		
	Time in culture (hours)				Time in culture (hours)		
	4	24	48		4	24	48
9	332	1797	342	2/7	500	1575	308
10	504	1989	245	3/7	590	2318	315
11	521	1920	160	4/7	606	2045	559
mean	452	1902	249	mean	565	1979	394
±SE	±61.5	±57.2	±53.5	±SE	±33.5	±221.1	±84.1

[H^3]thymidine incorporation was increased in lymphocytes from both normal and ^{131}I exposed rats, when tested 24 hours after commencement of culture with thyrocytes. Incorporation had decreased to earlier levels by 48 hours.

Table 6.

[H³] thymidine incorporation by lymphocytes (mean CPM)					
Normal rats	NLC[1]	NLCS[2]	[131]I exposed rats	AILC[3]	AILCS[4]
9	254	1797	2/7	403	1575
10	161	1989	3/7	234	2318
11	182	1920	4/7	181	2045
mean ±SE	199±28.6	1902±57.1	mean ±SE	272±68.2	1979±221.1

Note: **1.** NLC: Normal lymphocytes. These cells were derived from normal DA rats and were cultured alone.

2. NLCS: Normal lymphocytes stimulated by normal, syngeneic thyrocytes *in vitro.*

3. AILC: Autoimmune lymphocytes. These cells were derived from [131]I exposed DA rat and were cultured alone.

4. AILCS Autoimmune lymphocytes stimulated by normal, syngeneic thyrocytes *in vitro.*

Figure 21.

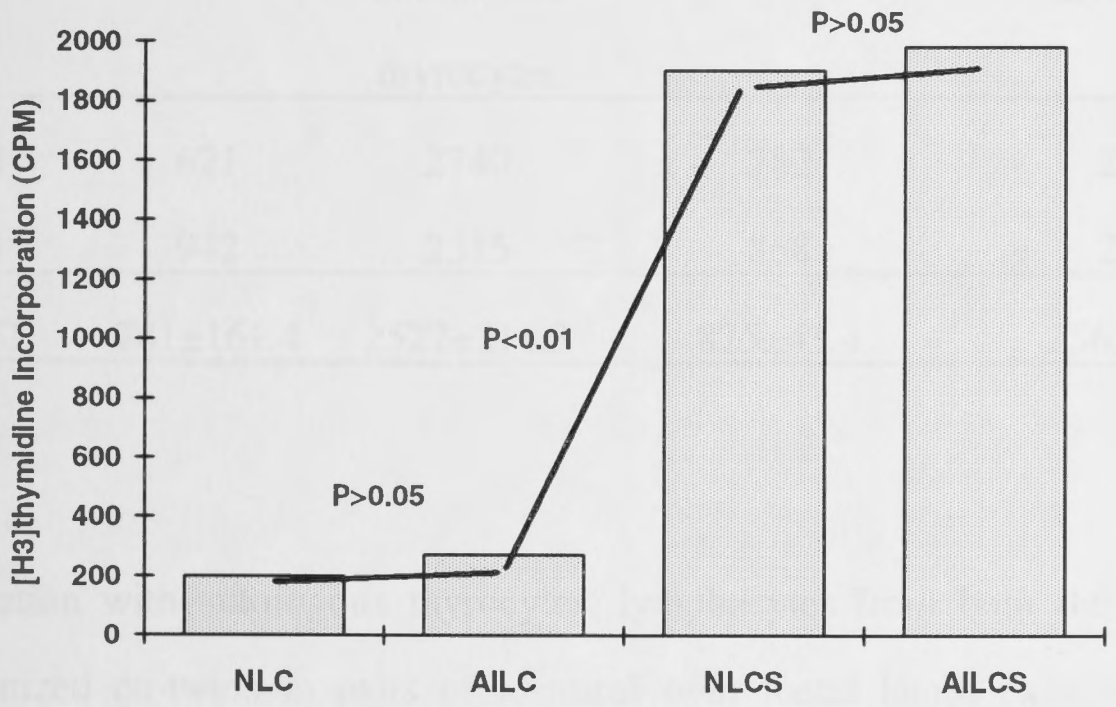


Table 6. and Figure 21. Comparison of [H³] thymidine incorporation by lymphocytes from normal and [131]I exposed DA rats determined 24 hours after initiation of incubation with syngeneic thyrocytes. Lymphocytes from both normal and [131]I exposed rats displayed a statistically significant increment ($P<0.01$) in the mean value (1902±57.1 CPM and 1979±221.1 CPM respectively) of [H³] thymidine incorporation after 24 hour incubation with syngeneic thyrocytes in comparison with the mean values for lymphocytes cultivated alone (mean values of 199±28.6 CPM and 272±68.2 CPM respectively). There was no significant difference between the mean values for [H³]thymidine incorporation of lymphocytes from normal and [131]I exposed rats after incubation with syngeneic thyrocytes.

Table 7. Comparison of [H^3] thymidine incorporation by lymphocytes from normal and thyroidectomized co-twins in pairs of identical twin foetal lambs.

	[H^3]thymidine incorporation of lymphocytes (mean CPM)			
Ewe bearing identical twin foetuses	Normal co-twin lymphocytes cultured alone	Normal co-twin lymphocytes cultured with autologous thyrocytes	Thyroidectomized co-twin lymphocytes cultured alone	Thyroidectomized co-twin lymphocytes cultured with autologous thyrocytes
Or783	621	2740	782	2641
Or826	942	2315	868	2485
mean ±SE	781±161.4	2527±214.3	825±43.4	2563±78.8

After incubation with autologous thyrocytes, lymphocytes from both the normal and thyroidectomized co-twins in pairs of identical twin foetal lambs exhibited levels of [H^3]thymidine incorporation considerably in excess of those of normal cells. However, there was no significant difference between the mean values of [H^3] thymidine incorporation by normal lymphocytes and lymphocytes from thyroidectomized foetal lambs.

Table 8. Comparison of the influence of numbers of autologous lymphocytes on the grade of damage produced in thyrocyte monolayers from thyroidectomized foetal lambs.

Ewe bearing thyroidectomized foetus	Grade of damage to thyrocyte monolayer: Lymphocyte numbers ($\times 10^6$)				
	2	5	7	10	12
Or30	1	2	4	4	4
Or230	1	4	4	5	5
Or235	1	4	5	5	5
W195	1	2	4	4	4
W208	1	4	4	5	5

Six different doses of lymphocytes from thyroidectomized foetal lambs were separately added to replicate cultures containing 10^6 autologous thyrocytes. The outcome of coculture of lymphocytes and thyrocytes was assessed 10 days later. There was no destruction of cultivated thyrocytes incubated with 2×10^6 lymphocytes. Three out of five cases in which thyrocytes were incubated with 5×10^6 autologous lymphocytes showed monolayer damage. Incubation with $7\text{-}12\times10^6$ lymphocytes produced severe damage to thyrocyte monolayers in all cases. As indicated in section 4.3.1 and illustrated in figure 1, the addition of 15×10^6 autologous lymphocytes from either a thyroidectomized or an intact foetus resulted in detachment of the monolayer from its coverslip. When this occurred in response to lymphocytes from an intact foetus, the cytotoxic changes in follicles and individual thyrocytes, observed on exposure to lymphocytes from thyroidectomized foetuses, were lacking.

Table 9. The influence of culture time on the degree of damage to autologous thyrocyte monolayers achieved by lymphocytes from thyroidectomized foetal lambs.

Ewe bearing thyroidectomized foetus	Grade of damage to thyrocytes monolayer			
	Time in culture (days)			
	2	5	7	10
Or30	0	2	3	4
Or230	1	3	4	5
Or235	0	2	4	5
W195	0	2	3	4
W208	0	3	4	5
P30	0	3	4	5

Six thyroidectomized foetal lambs were used to examine the influence of culture time on the occurrence of thyrocyte destruction after incubation with autologous lymphocytes. The 4 different culture times tested were 2, 5, 7 and 10 days. No destruction of thyrocytes was evident after 2 days incubation. Thyrocytes had sustained moderate destruction in 3 out of 6 cases while the remaining cases retained a normal thyrocyte structure at 5 days of incubation. In all cases tested, damage to the thyrocytes was evident by 7 days incubation, but in 2 out of 6 cases, this damage remained only moderate. However, severe destruction of thyrocytes was observed in all cases after 10 days of incubation.

Table 10. The influence of lymphocytes from ^{131}I exposed DA rats, stimulated in vivo by syngeneic thyroid implantation, on the degree of damage produced in cultivated thyrocytes

^{131}I exposed DA rats	Grade of damage of thyrocyte monolayer before thyroid challenge *in vivo* (10 days)	Grade of damage of thyrocyte monolayer after thyroid challenge *in vivo* (10 days)
1/1	2	2
2/1	2	2
3/1	2	3
4/1	5	5
1/5	5	5
2/5	5	5
1/6	2	2
1/7	2	2
2/7	3	4
3/7	3	4
4/7	3	4
1/4	3	4
2/4	3	4
1/9	3	4
2/9	3	4
1/11	4	4
2/11	3	4
1/12	3	4
2/12	2	4
1/10	3	4
1/13	4	4
2/13	4	4
3/13	4	4

In all 10 cases in which there was only moderate damage (grade 3) and in 2 out of 6 cases in which normal follicle structure was retained (grade 2) following cultivation with lymphocytes from ^{131}I exposed rats *before* challenge, the level of cytotoxic damage increased when lymphocytes from the same rats were re-tested after challenge of the donor by means of a syngeneic thyroid implant.

Table 11. Comparison of the cytotoxicity for syngeneic thyrocytes of lymphocytes from different lymphoid organs of ^{131}I exposed rats.

	Normal rats		^{131}I exposed rats	
	Time in culture (days)		Time in culture (days)	
	5	10	5	10
Spleen cells	4	5	4	5
Lymph node cells	0.2	1.2	2	4
Thymus cells	0	1	0.5	1

In these experiments, 8 normal and 11 ^{131}I exposed DA rats have been tested. In all cases, thyrocyte monolayers were incubated with 10^7 lymphocytes. Thyrocytes showed extensive destruction (grade 4) at an earlier incubation time (5 days) after incubation with spleen lymphocytes, rather than lymph node lymphocytes, from either 8 normal or 11 ^{131}I exposed DA rats. In contrast, thyrocytes were unaffected after incubation with thymus or lymph node lymphocytes from 8 normal rats or with thymus cells from 11 ^{131}I exposed rats. After incubation with lymph node cells from ^{131}I exposed rats, (but not from normal rats) moderate or severe destruction of thyrocytes became apparent by 10 days of incubation.

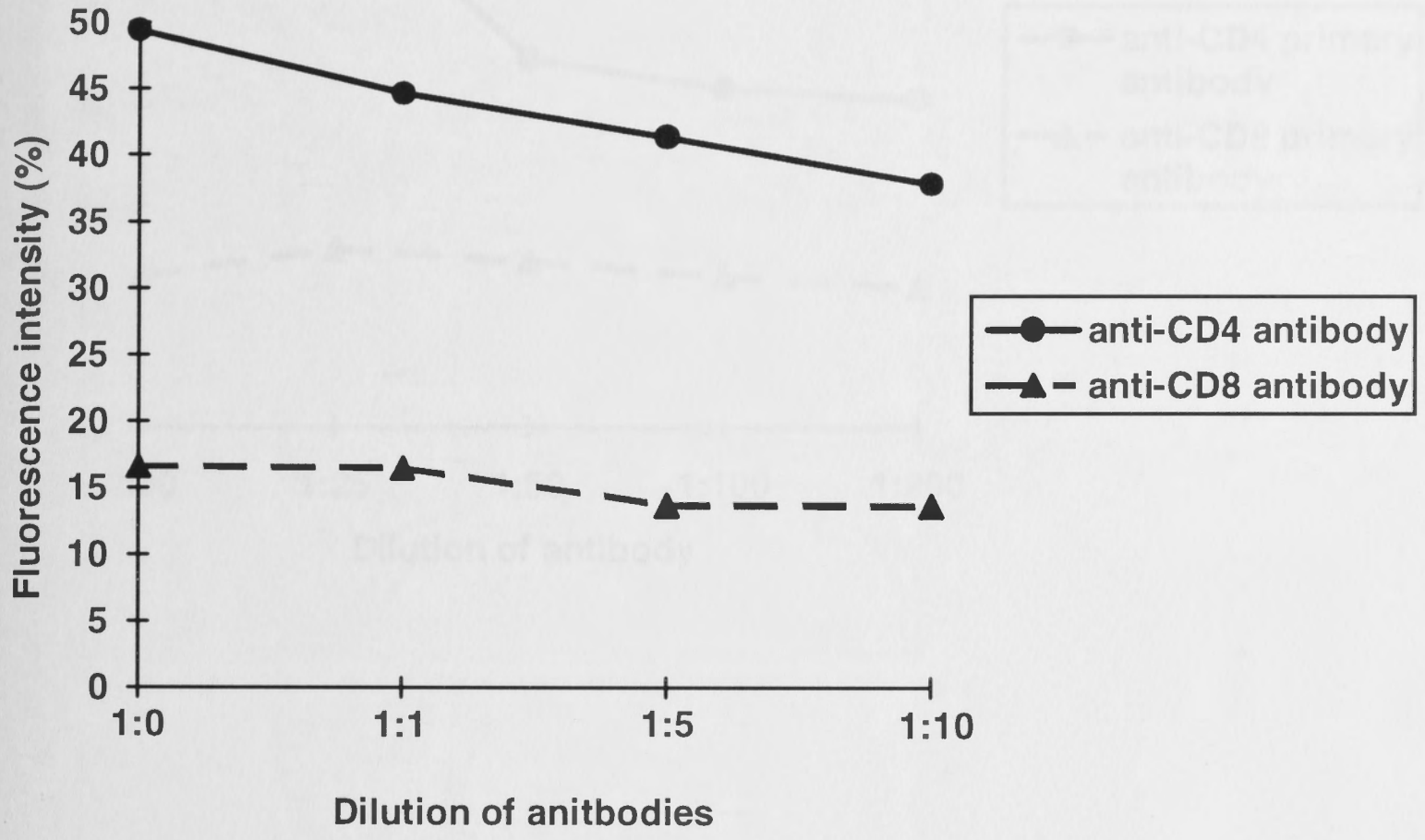


Figure 22. Dilution of primary antibodies. Appropriate dilutions of primary antibodies have been determined by antibody titration against a fixed number of cells. A fixed numbers of 10^6 lymphocytes from the normal identical co-twin of foetal lamb were incubated with 100µl of either anti-CD4 or anti-CD8 primary antibody in the following dilutions: 1:0 (undiluted) 1:1, 1:5 and 1:10. The point at which saturation of binding sites occurred was defined on the flow cytometer when increasing antibody concentration produced little increase in fluorescence intensity. The cells stained with undiluted antibody (either anti-CD4 or anti-CD8) displayed the highest intensity of positive fluorescence, implying that this was a saturating dilution.

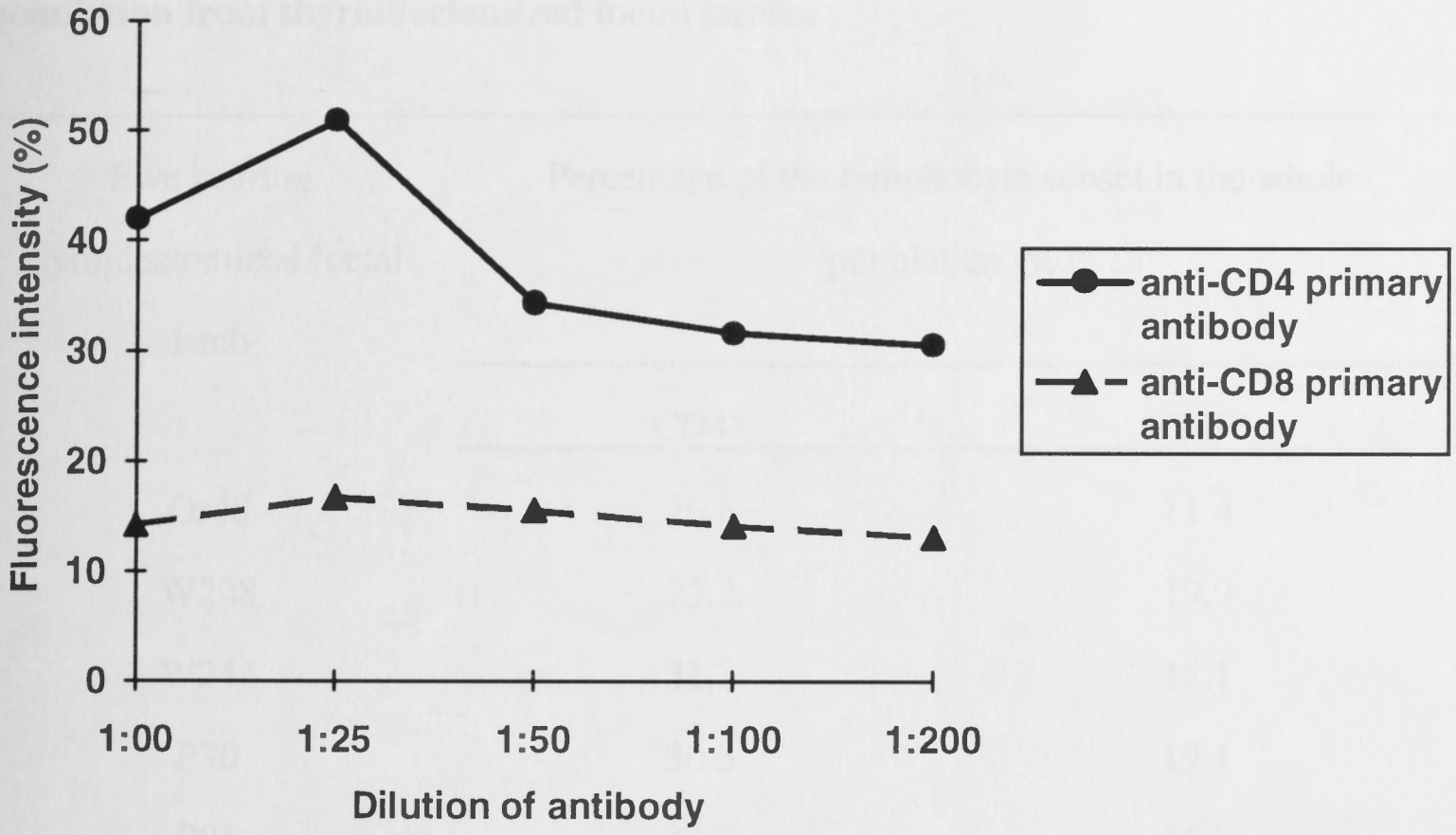


Figure 23. Dilution of second antibody. At a fixed dilution (undiluted) of primary antibody (either anti-CD4 or anti-CD8), 10^6 lymphocytes from the normal identical co-twin of foetal lamb were then incubated with 100µl of secondary antibody (rabbit anti-mouse IgG antibody conjugated with fluoresceinisothiocyanate FITC) in the following dilutions: undiluted, 1:25, 1:50, 1:100 and 1:200. Cells stained with this secondary antibody at a 1:25 dilution manifested the highest intensity of positive fluorescence. This denoted that a 1:25 dilution of this secondary antibody was a saturating dilution. The 1:50 dilution used in the present experiments was not a saturating dilution.

Table 12. Percentage of $CD4^+$ or $CD8^+$ subset cells in the whole lymphocyte population from thyroidectomized foetal lambs.

Ewe bearing thyroidectomized foetal lamb	Percentage of the lymphocyte subset in the whole population (%)	
	$CD4^+$	$CD8^+$
Or48	29.6	11.4
W208	23.2	19.9
W244	31.3	18.1
P30	30.3	19.1
P91	43.2	16.0
mean ±SE	31.5±3.3	16.9±1.5

Percentage of the $CD4^+$ and $CD8^+$ lymphocyte subsets were determined by flow cytometry. There was a mean 31.5%±3.3% $CD4^+$ subset cells and a mean 16.9%±1.5% $CD8^+$ subset cells in the whole lymphocyte population from thyroidectomized foetal lambs.

Table 13. The effect of co-culturing autologous thyrocytes with lymphocyte subpopulations from thyroidectomized foetal lambs.

Ewe bearing thyroidectomized foetal lamb	Grade of cytotoxicity for autologous thyrocytes of lymphocyte subsets from thyroidectomized foetus				
	whole lymphocyte population	CD4 selected	CD4 depleted	CD8 selected	CD8 depleted
Y289	5	2	4	3	2
Y297	4	2	4	2	2
Y277	4	2	NT[1]	2	NT
W216	4	2	4	NT	NT
Y292	5	2	4	NT	NT
Y303	4	2	4	NT	NT
P30	5	2	4	NT	NT
Y319	5	NT	NT	2	2
P91	4	NT	NT	2	2

Note 1. not tested.

Lymphocytes from 9 thyroidectomized foetal lambs have been examined. Severe damage to thyrocytes, comparable with that produced by whole lymphocyte populations, was observed only after incubating them with the CD4 depleted population.

Figure 24. Experimental plan 3.

(1) One pair of identical twin foetal lambs of 51-54 days gestation was submitted to bilateral thyroidectomy.

(2) Excised thyroid glands were converted into single cell suspensions and stored at -196°C for more than 8 weeks.

(3) Cryopreserved thyrocytes were thawed and cultured for 3 days before the addition of lymphocytes.

(4) At 100-125 days of gestation, lymph nodes from both identical twin foetuses were removed and converted into single cell suspensions.

(5) A mixture of lymphocytes from the normal foetus and its thyroidectomized identical co-twin were cultured with autologous thyrocytes from the thyroidectomized co-twin.

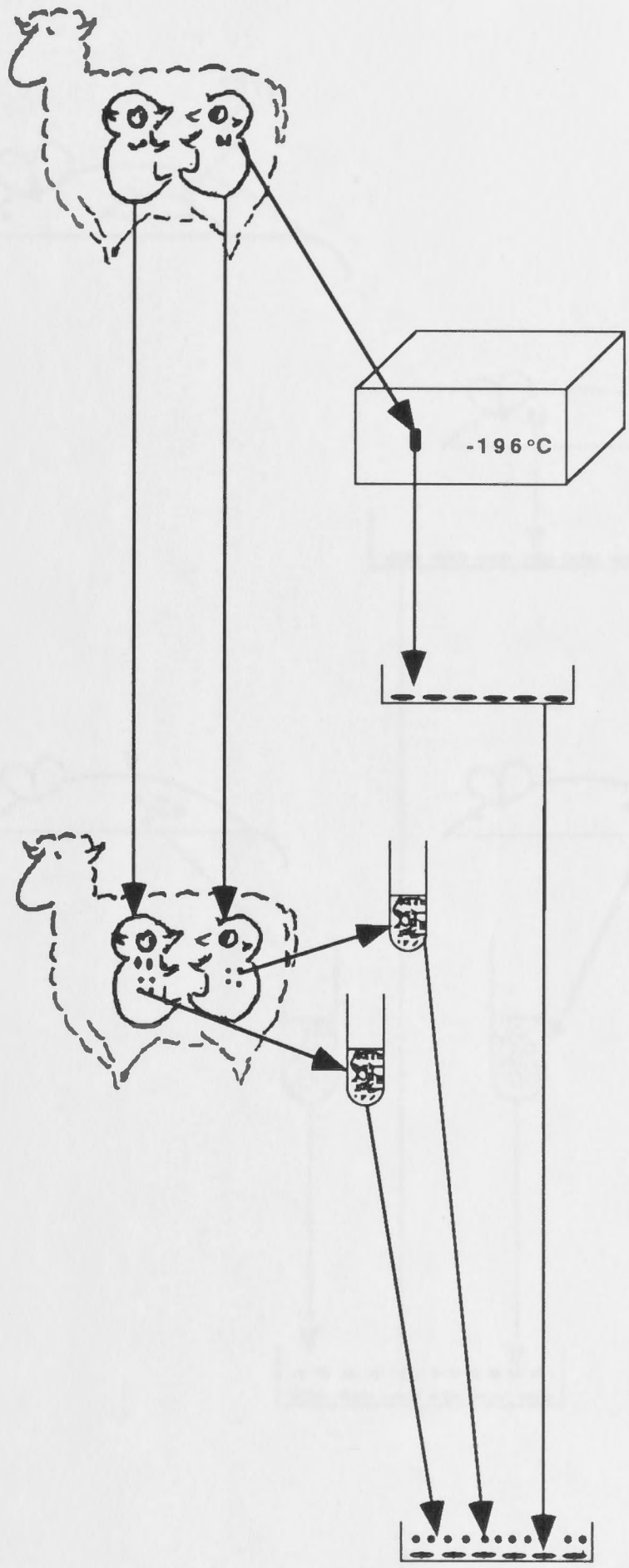


Figure 25. Experimental plan 4.

(1) At 17 days of gestation, foetal DA rats received a dose of ^{131}I sufficient to disrupt further developement of the thyroid gland.

(2) Thyroid glands from syngeneic neonatal DA rats were removed and converted into single cell suspensions which were then cultured for 5 days before incubation with lynphocytes.

(3) Lymph nodes were removed from normal and ^{131}I exposed 1 year old DA rats and converted into single cell suspensions.

(4) A mixture of freshly isolated lymphocytes from both types of rat were then incubated with syngeneic thyrocytes that had been previously cultured for 5 days.

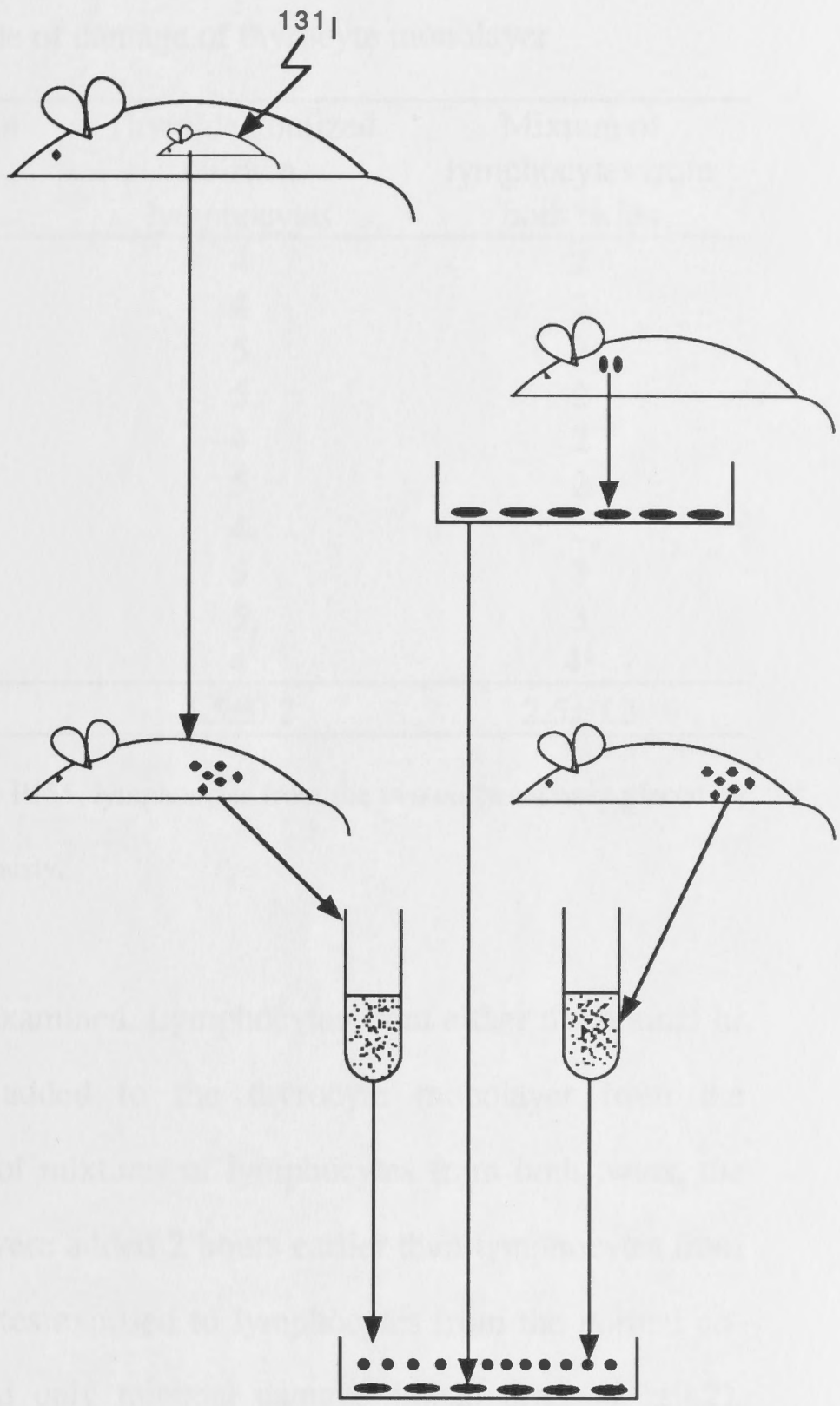


Table 14. The effect of lymphocytes from normal and thyroidectomized identical twin foetal lambs on autologous thyrocyte monolayers.

Ewe bearing identical twin foetuses	Grade of damage of thyrocyte monolayer		
	Normal co-twin lymphocytes	Thyroidectomized co-twin lymphocytes	Mixture of lymphocytes from both twins
Or783	2	4	2
Or849	1	4	2
W76	2	5	3
W69	1	5	2
P8	1	4	2
P78	1	5	2
P91	1	4	2
P118	1	5	3
P138	1	5	3
P155	2	4	4[1]
mean±SE	1.3±0.2	4.5±0.2	2.5±0.2

Note: 1. In the case of the pair of twins in ewe P155, lymphocytes from the two co-twins were placed on the autologous thyrocyte monolayer simultaneously.

Ten pairs of identical twins have been examined. Lymphocytes from either the normal or the thyroidectomized co-twin were added to the thyrocyte monolayer from the thyroidectomized co-twin. In the case of mixtures of lymphocytes from both twins, the lymphocytes from the normal co-twin were added 2 hours earlier than lymphocytes from the thyroidectomized co-twin. Thyrocytes exposed to lymphocytes from the normal co-twin remained unaffected or displayed only minimal damage (mean grade 1.3±0.2). However, severe damage (mean grade 4.5±0.2) followed incubation of thyrocytes with autologous lymphocytes from the thyroidectomized foetal co-twin in all 10 cases. After incubation with the lymphocytes from both co-twins, damage to thyrocytes was substantially curtailed in 9 out of 10 instances (mean grade 2.5±0.2). The mean grade (2.5) of damage to the thyrocyte monolayers incubated with a mixture of lymphocytes was significantly lower ($P<0.01$) than the mean grade (4.5) induced by the lymphocytes from thyroidectomized co-twin.

Figure 26. The curtailment of cytotoxic responses against autologous thyrocytes by lymphocytes from a thyroidectomized foetal lamb as a result of co-cultivation with lymphocytes from its normal, identical co-twin.

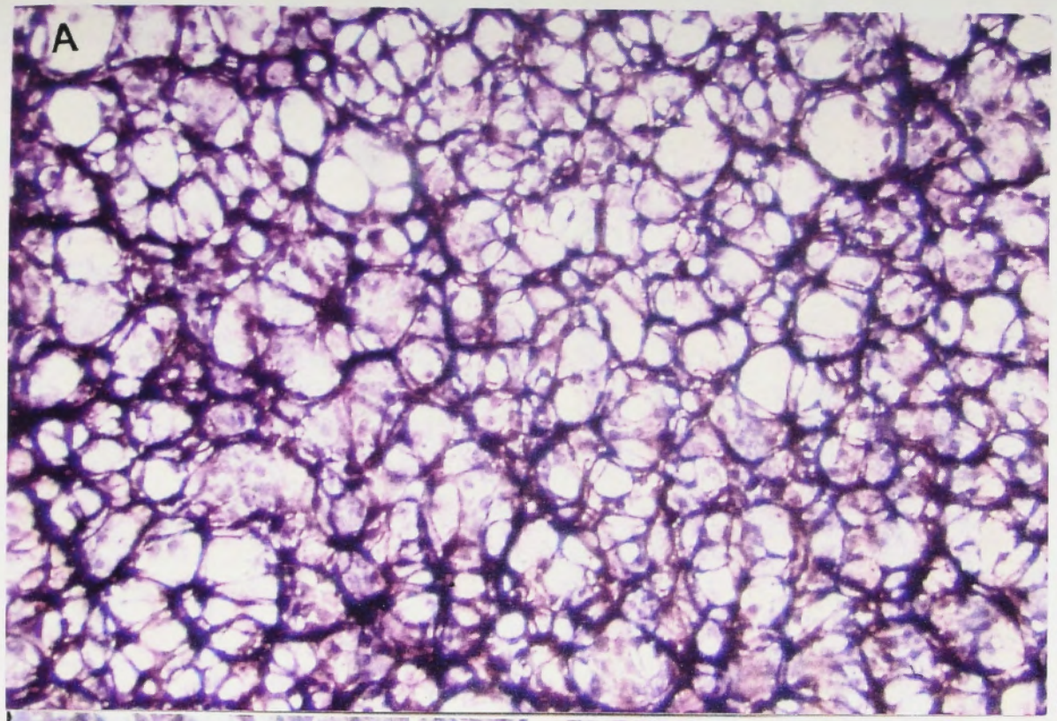
(All results in this figure were obtained from the pair of identical twins of ewe P91. Fixation of specimens was performed 10 days after adding lymphocytes to thyrocyte monolayers. The coverslips bearing the indicated cells were all stained with H&E. Magnification was ×156.)

(A) *Thyrocytes cultivated alone.* Thyrocyte follicles remained intact and healthy. (Grade 0).

(B) *Thyrocytes cultivated with 7× 10^6 lymphocytes from normal co-twin.* Thyrocyte follicles remained intact in most areas of the monolayer. (Grade 1).

(C) *Thyrocytes cultivated with 5× 10^6 lymphocytes from thyroidectomized co-twin.* The thyrocyte monolayer was severely damaged. (Grade 4).

(D) *Thyrocytes cultivated with a mixture of 7× 10^6 lymphocytes from the normal and 5× 10^6 lymphocytes from the thyroidectomized co-twin.* Most follicle epithelial cells retained their distinct structure. Foci of slight damage to thyroid follicles were occasionally found. (Grade 2).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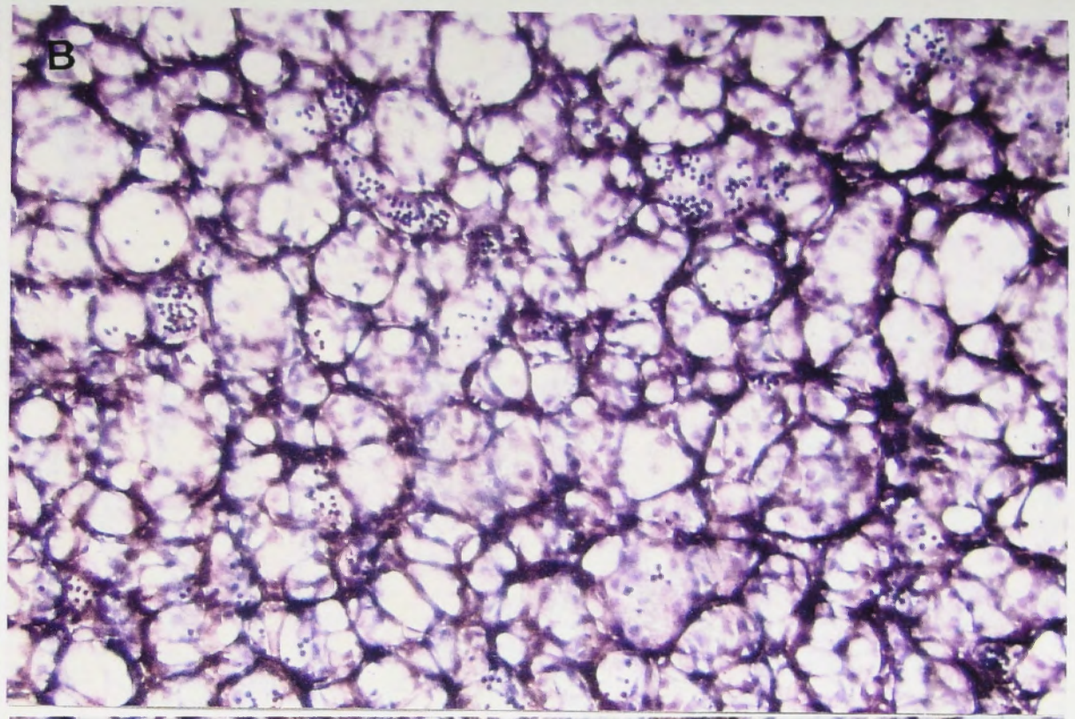
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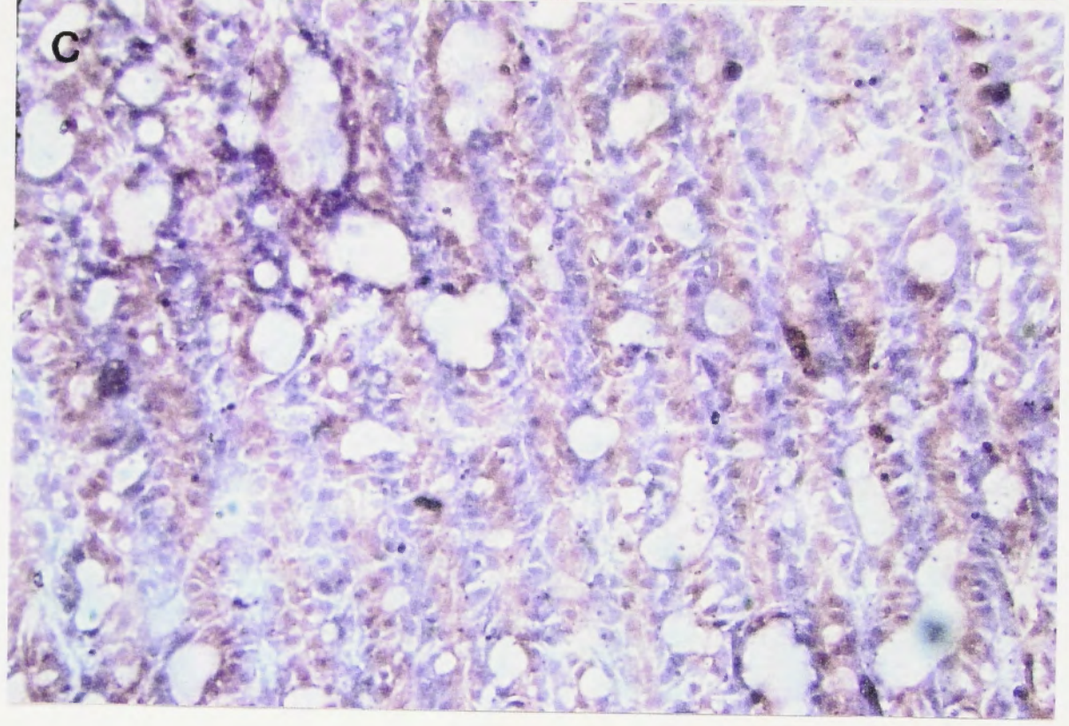
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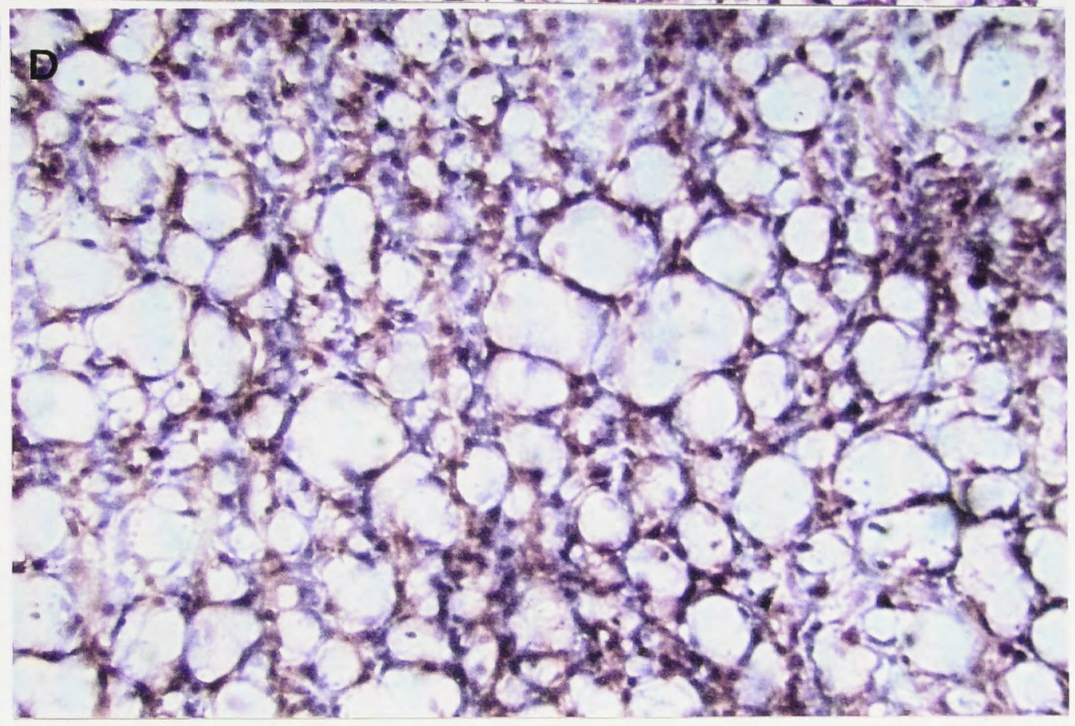
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Table 15. The effect of lymphocytes from normal and ^{131}I exposed DA rats on syngeneic thyrocyte monolayers.

^{131}I exposed rat	Grade of damage to thyrocyte monolayer			
	Normal rat lymphocytes	^{131}I exposed rat lymphocytes	Mixture of lymphocytes from ^{131}I exposed and normal rat	Lymphocyte numbers (normal:^{131}I-exposed rat) ($\times 10^6$)
4/1	1	5	2	5:5
1/5	1	5	2	5:5
2/5	1	5	3	5:5
2/7	1	4	3	5:5
3/7	1	4	4	5:5
4/7	1	4	4	5:5
1/4	2	4	2	5:5
1/9	1	4	4	5:5
1/11	1	4	2	7:5
2/11	1	4	2	7:5
1/13	1	4	2	7:5
2/13	1	4	2	7:5
mean±SE	1.1±0.1	4.3±0.1	2.6±0.3	

The effect of lymphocytes from 12 ^{131}I exposed and 12 normal DA rats was tested on syngeneic thyrocyte monolayers. Incubation with lymphocytes from ^{131}I exposed rats that had previously received implants of syngeneic thyroid resulted in severe damage to thyrocytes (mean grade 4.3±0.1). However, thyrocytes retained their normal structure after incubation with lymphocytes from normal syngeneic rats (mean grade 1.1±0.1). After incubation with mixtures of lymphocytes from both ^{131}I exposed and normal rats, damage to thyrocyte monolayers was curtailed in 9 out of the 12 cases tested. Addition of 5×10^6 normal lymphocytes to 5×10^6 cells from ^{131}I exposed rats curtailed autoreactivity in 5 out of 8 cases. 7×10^6 normal lymphocytes were effective in all 4 instances. The mean grade (2.6±0.3) induced by a mixture of lymphocytes was significantly lower ($P<0.01$) than the mean grade of 4.3±0.1 induced by lymphocytes from ^{131}I exposed rats.

Figure 27. The curtailment of cytotoxic responses against syngeneic thyrocytes by lymphocytes from ^{131}I exposed rats as a result of co-cultivation with normal, syngeneic DA lymphocytes.

(The results in this figure were obtained using lymphocytes from ^{131}I exposed DA rat 1/13 and normal DA rat 21. Fixation of specimens was performed 10 days after adding lymphocytes to thyrocyte monolayers. The coverslips bearing the indicated cells were all stained with H&E. Magnification ×244.)

(A) *Cultivated thyrocytes alone.* Thyrocyte follicles remained intact and healthy. (Grade 0).

(B) *Thyrocytes cultivated with 7× 10^6 syngeneic lymphocytes from normal DA rat 21.* Thyrocyte follicles remained intact in most areas of the monolayer. (Grade1).

(C) *Thyrocytes cultivated with 5× 10^6 syngeneic lymphocytes from ^{131}I exposed DA rat 1/13* . The thyrocyte monolayer was severely damaged. (Grade 4).

(D) *Thyrocytes cultivated with a mixture of 7× 10^6 lymphocytes from normal rat 21 and 5× 10^6 lymphocytes from ^{131}I exposed rat 1/13.* Most follicle epithelial cells retained their distinct structure. Foci of slight damage to thyroid follicles were occasionally found. (Grade 2).

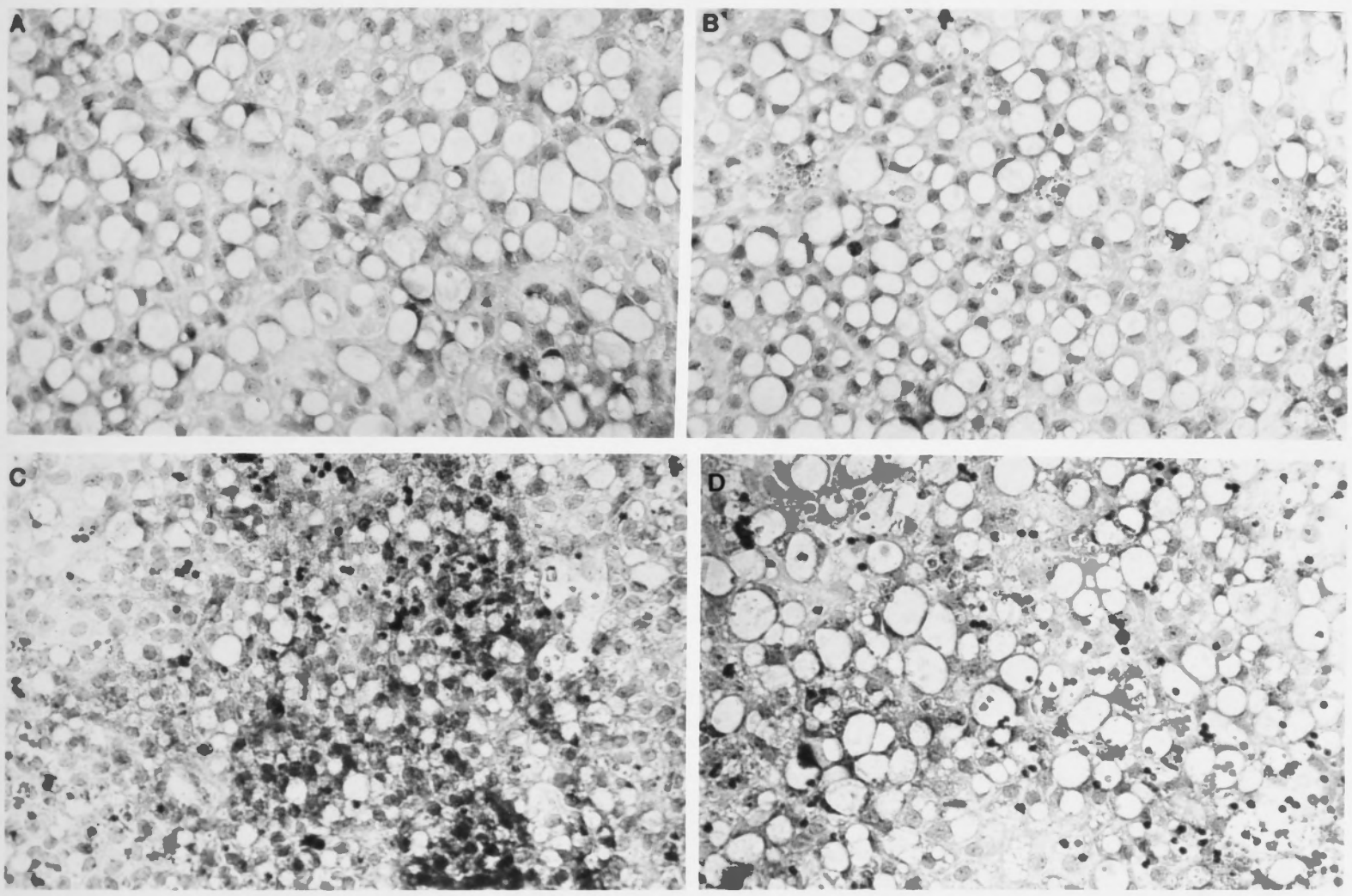
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Table 16. The influence of the ratio of lymphocytes from normal and thyroidectomized foetal lamb co-twins on curtailment of cytotoxicity.

Ewe bearing identical twin foetuses	Grade of damage to thyroid monolayer after incubation with different numbers of lymphocytes from the two co-twins (thyroidectomized foetus:normal foetus) ($\times 10^6$)		
	5:0	5:5	5:7
Or783	4	4	2
Or849	4	3	2
W69	5	3	2
W76	5	4	3

As 5×10^6 lymphocytes from thyroidectomized foetal lambs regularly produced severe damage to thyrocyte monolayers, this number of cells was selected as the standard to test the capacity of normal lymphocytes to interfere with cytotoxicity. Different numbers of lymphocytes from normal individuals were incubated with lymphocytes from thyroidectomized foetuses. 7×10^6 lymphocytes from the normal, identical co-twin foetus curtailed the autoreactivity of lymphocytes from the corresponding thyroidectomized co-twin in all cases. Exposure of monolayers to a mixture of 5×10^6 normal and 5×10^6 autoreactive lymphocytes resulted in curtailment of cytotoxic response in 2 out of 4 cases tested.

Table 17. The influence on curtailment of cytotoxicity of the interval elapsing between placement of normal lymphocytes on autologous thyrocyte monolayers and the introduction of lymphocytes from thyroidectomized foetuses.

Ewe bearing identical twin foetuses	Grade of damage of thyrocyte monolayer		
	Lymphocytes from thyroidectomized co-twin alone	Simultaneous placement of lymphocytes from both co-twins	Placement of normal lymphocytes 2 hours before cells from thyroidectomized foetus
Or783	4	NT[1]	2
Or849	4	NT	2
W76	5	NT	3
W69	5	NT	2
P8	4	NT	2
P78	5	3	2
P91	4	3	2
P118	5	4	3
P138	5	3	NT
P155	4	4	NT

Note: 1. Not tested.

Curtailment of damage to thyrocyte monolayers occurred in all 8 cases tested when normal lymphocytes were placed on thyrocyte monolayers 2 hours earlier than lymphocytes from the thyroidectomized foetus. When lymphocytes from the 2 co-twins were simultaneously added, curtailment of damage to thyrocytes was less effective.

Table 18. The influence on curtailment of cytotoxicity of the interval elapsing between placement of normal DA rat lymphocytes on syngeneic thyrocyte monolayers and the introduction of lymphocytes from ^{131}I exposed rats.

^{131}I exposed rat	Grade of damage to thyrocyte monolayer		
	Lymphocytes from ^{131}I exposed rats alone	Simultaneous placement of lymphocytes from normal and ^{131}I exposed rats	Placement of normal lymphocytes 2 hours before cells from ^{131}I exposed rat
4/1	5	5	2
1/5	5	4	2
2/5	5	4	3
1/13	4	3	2
2/13	4	3	2
1/11	4	4	2

When normal lymphocytes and lymphocytes from ^{131}I exposed DA rats were simultaneously added to DA thyrocyte monolayers, there was a lack of curtailment of autoimmune cytotoxicity in 4 out of 6 cases. However, placement of normal lymphocytes on the thyrocyte monolayers 2 hours before introduction of lymphocytes from ^{131}I exposed rats effectively curtailed the damage in all cases.

Table 19. Percentage of $CD4^+$ and $CD8^+$ subsets in whole populations of lymph node cells from normal identical co-twins.

Ewe bearing identical twin	Percentage of subpopulation cells in whole population of lymphocytes from normal co-twin	
	$CD4^+$	$CD8^+$
P6	19.9	14.4
P8	19.7	13.2
P91	51.3	14.7
W69	33.2	16.6
W140	37.9	19.4
mean±SE	32.4±6	15.7±1

$CD4^+$ subset cells were a mean 32.4±6% and $CD8^+$ subset cells constituted 15.7±1% of the whole population of 10^6 lymphocytes from normal co-twin foetus.

Table 20. Recovery rate and death rate of lymphocytes from normal identical co-twins after sorting by flow cytometry.

Ewe bearing foetal lamb	Lymphocytes before sorting ($\times 10^6$)	Lymphocytes after sorting ($\times 10^6$)	Recovery rate (%)	Non viable cells among recovered lymphocytes (%)
W113	25	17	68	3
Or849	25	17.5	70	4
P8	25	17	68	4
P118	25	18.8	75	5
mean	25	17.6	70	4

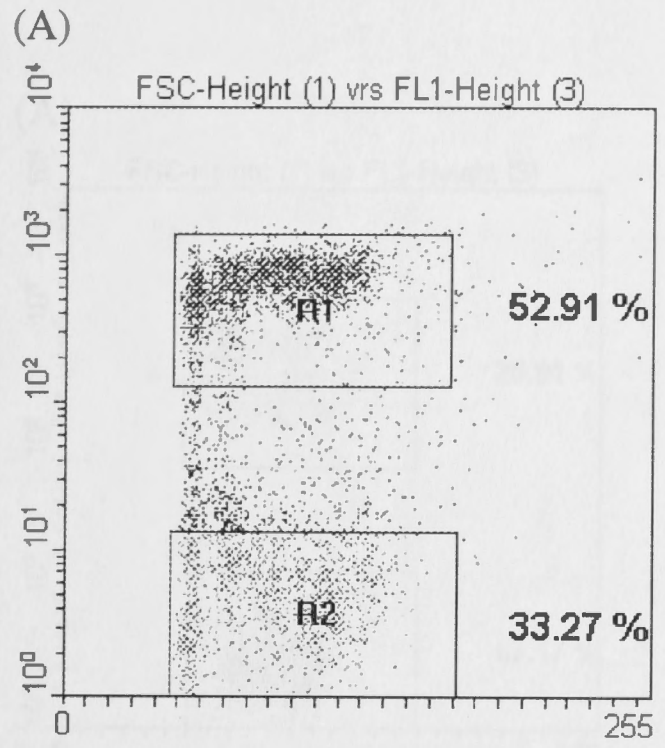
After sorting by flow cytometry, 70% of the initial number of lymphocytes from normal identical co-twins were recovered and 96% of these were viable.

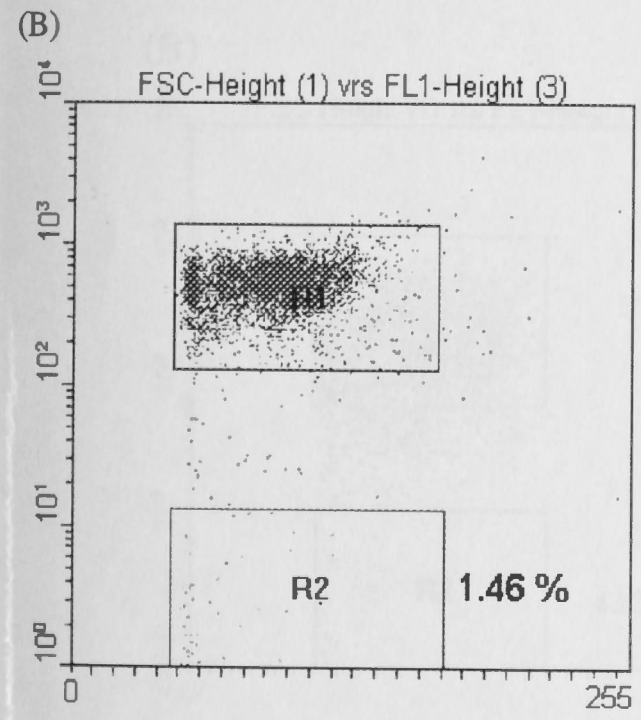
Table 21 The purity of lymphocytes from normal identical co-twins after sorting by flow cytometry.

Ewe bearing foetal lamb	Purity of lymphocytes after sorting by flow cytometry (%)			
	CD4 selected cells	CD4 depleted cells	CD8 selected cells	CD8 depleted cells
W113	98.5	98.9	95.8	99.7
Or849	97.3	99.5	98.2	99.9
P8	97.2	98.9	97.5	98.6
P118	NS[1]	NS	96.4	99.8
mean±SE	97.6±0.4	99±0.2	97±0.5	99.5±0.3

Note: 1. Not sorted.

The purity of sorted cell populations was assessed by running small samples of the sorted population through the instrument using the same windows. The purity was then expressed as the percentage of cells falling in the sort gate for the desired cell population, exclusive of the percentage of cells falling in the sort gate for the cells that were not required. Mean value of purity in CD8 selected cells was 97%. The mean value in other 3 subpopulations was more than 97%.





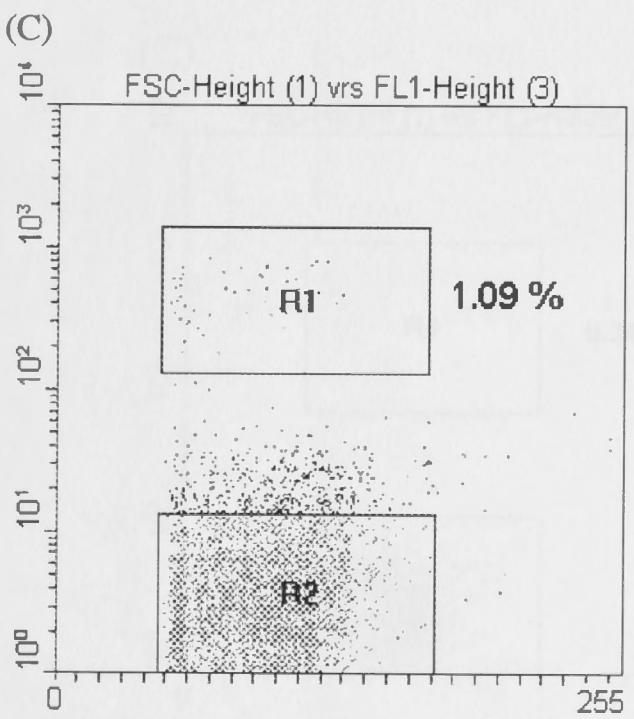
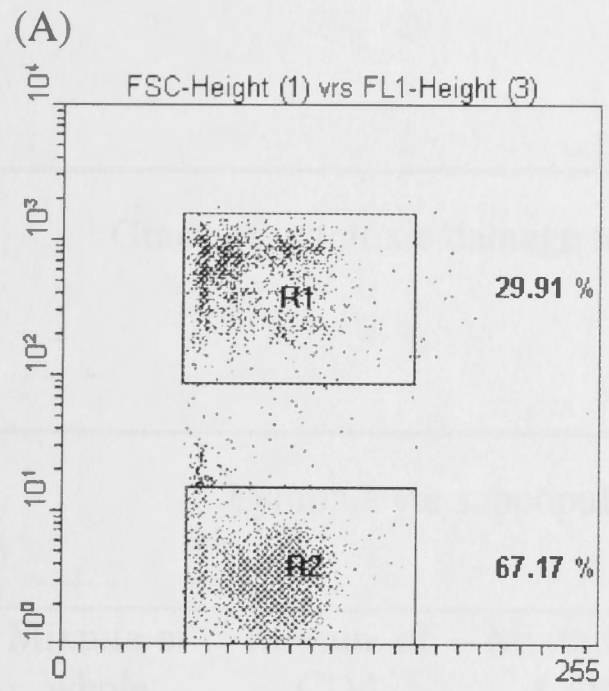
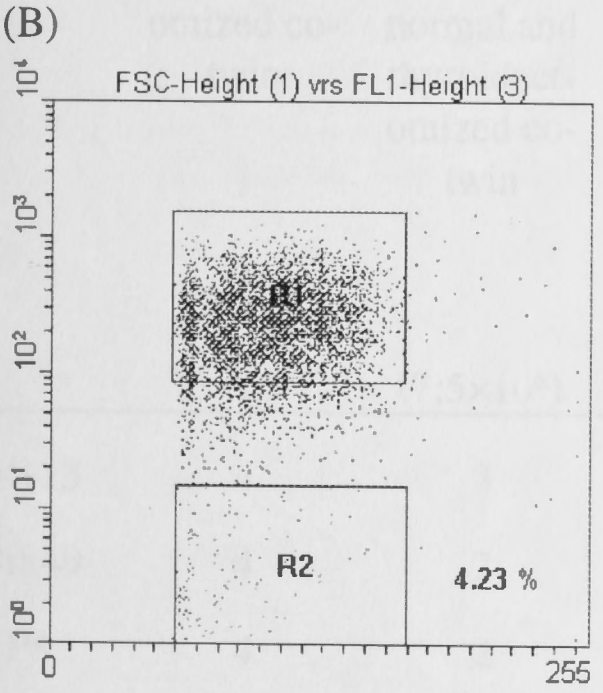


Figure 28. Purity of CD4 selected and CD4 depleted subpopulation after sorting by flow cytometry. (A) shows the percentage of $CD4^+$ and $CD4^-$ cells in a whole population of lymphocytes from a normal co-twin before sorting by flow cytometry. (B) shows purity of CD4 selected cells after sorting. (C) shows purity of CD4 depleted cells after sorting.





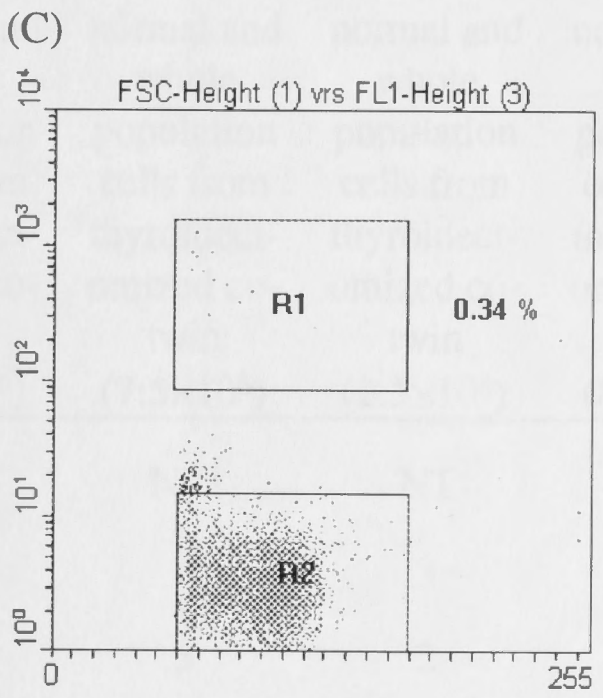


Figure 29. Purity of CD8 selected and CD8 depleted subpopulation after sorting by flow cytometry. (A) shows percentage of $CD8^+$ and $CD8^-$ cells in a whole population of lymphocytes from a normal co-twin before sorting by flow cytometry. (B) shows purity of CD8 selected cells after sorting. (C) shows purity of CD8 depleted cells after sorting.

Table 22. Influence of different T lymphocyte subsets from normal foetal co-twins on the cytotoxicity for autologous thyrocytes of lymphocytes from thyroidectomized foetal co-twins.

Ewe bearing identical twins	Grade of cytotoxic damage to thyrocytes: Lymphocyte subpopulation					
	Whole population cells from thyroidectomized co-twin (5×10^6)	Mixture of whole population cells from normal and thyroidectomized co-twin ($7{:}5\times10^6$)	Mixture of CD4 selected cells from normal and whole population cells from thyroidectomized co-twin ($3{:}5\times10^6$)	Mixture of CD4 depleted cells from normal and whole population cells from thyroidectomized co-twin ($7{:}5\times10^6$)	Mixture of CD8 selected cells from normal and whole population cells from thyroidectomized co-twin ($2{:}5\times10^6$)	Mixture of CD8 depleted cells from normal and whole population cells from thyroidectomized co-twin ($8{:}5\times10^6$)
W113	4	3	4	NT[1]	NT	4
Or849	4	2	NT	4	3	NT
P8	4	2	4	3	2	4
P118	5	3	NT	NT	3	4

Note: **1.** Not tested.

After lymphocyte sorting, four lymphocyte subpopulations were obtained. Only that subpopulation of lymphocytes from the normal co-twin selected for expression of CD8 effectively curtailed autoreactivity on the part of cells from the thyroidectomized co-twin.

Table 23. The influence of thymectomy of thyroidectomized foetal lambs on the occurrence of autoreactivity by their lymphocytes against cultivated autologous thyrocytes.

Ewe bearing thyroidectomized foetus	Grade of damage to thyrocyte monolayer		Ewe bearing thyroidectomized-thymectomized foetus	Grade of damage to thyrocyte monolayer	
	5 days	10 days		5 days	10 days
Or30	2	4	Y248	3	4
Or230	3	5	Y251	4	5
Or235	2	5	Y246	4	5
W195	2	4	Y265	4	5
W208	3	5	Y249	3	5
P30	3	5	Y250	4	5
mean±SE	2.5±0.2	4.6±0.2	mean±SE	3.7±0.2	4.8±0.2

Six foetuses were submitted to thymectomy together with thyroidectomy. Damage to thyrocytes in these cases occurred earlier than in foetuses thyroidectomized alone. At 5 days of incubation with autologous lymphocytes from thyroidectomized-thymectomized foetuses, thyrocytes had sustained severe damage in 4 out of 6 cases. Moderate damage to the thyrocyte monolayer had occurred in the 2 remaining cases. The mean grade of damage to the thyrocyte monolayer for this group was 3.7±0.2. In comparison, lymphocytes from foetuses which had only been thyroidectomized produced only moderate damage in 3 out of 6 cases and slight destruction in the remainder at 5 days of cultivation. The mean grade of damage to thyrocyte monolayers for this group was 2.5± 0.2. The damage produced by lymphocytes from thyroidectomized and thymectomized foetuses was significantly higher ($P<0.01$). Severe damage to thyrocyte monolayers had occurred in all cases of both groups by 10 days incubation.

Figure 30. Experimental plan 5.

(1) At 51-54 days of gestation, foetal lambs underwent bilateral thyroidectomy followed by the implantation of an allogeneic thyroid lobe from a foetal lamb of the same age.

(2) Two thyroid lobes removed from the recipient and one lobe removed from the donor were converted into single cell suspensions and were then stored at -196°C for 60-70 days.

(3) Cryopreserved thyrocytes were thawed and established as monolayers in culture before the introduction of lymphocytes.

(4) At 100-120 days of gestation, lymph nodes were removed from the thyroid allografted recipient foetus and a single cell suspension was prepared.

(5) Lymphocytes freshly isolated from the recipient were then incubated with thyrocyte monolayers from recipient or donor.

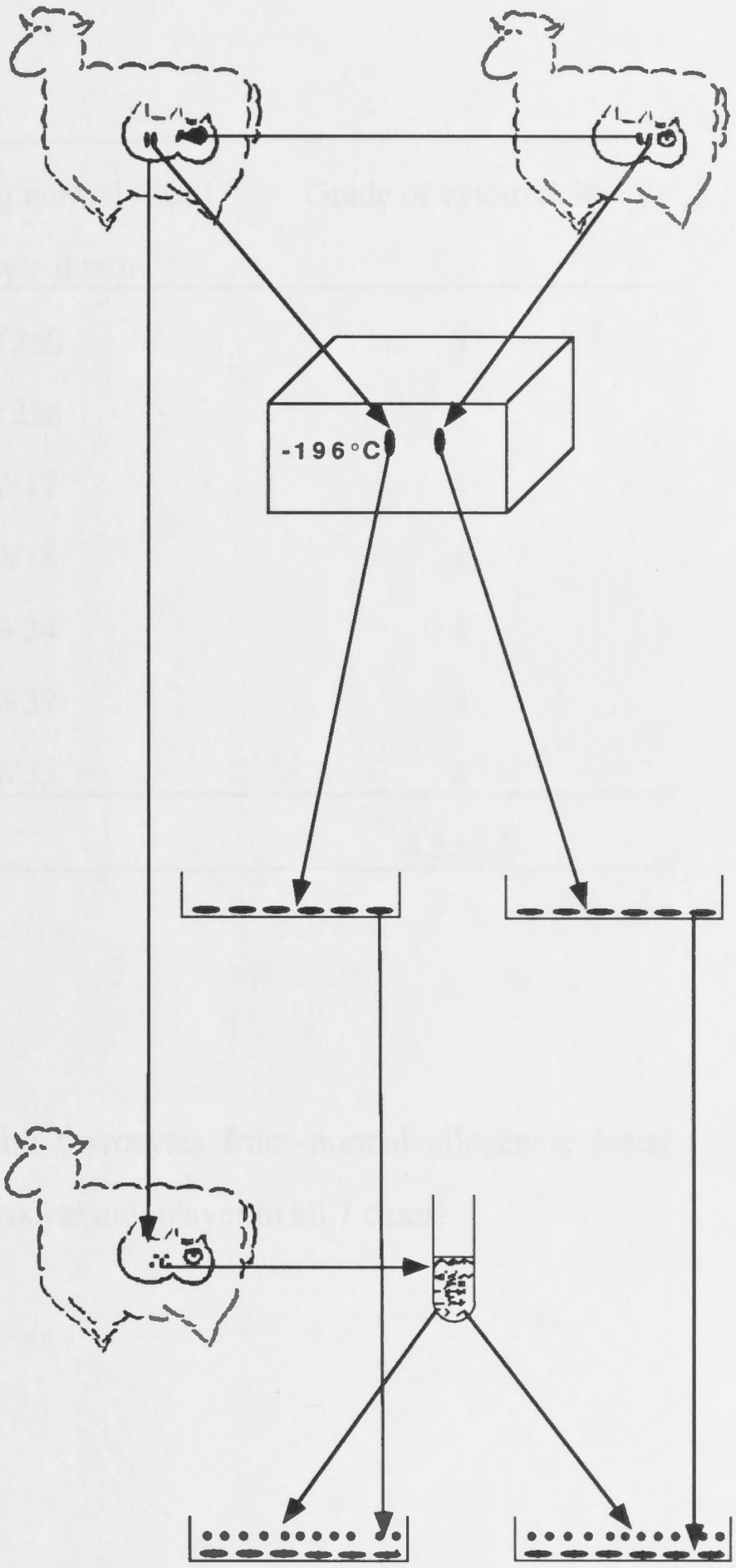


Table 24. The effect of co-cultivating thyrocytes from normal foetal lambs with lymphocytes from normal allogeneic foetal lambs.

Ewe bearing normal foetal lymphocyte donor	Ewe bearing normal foetal thyrocyte donor	Grade of cytotoxicity
Y261	Y260	5
Y261	Y256	5
W44	W17	4
W44	W18	4
W44	W24	4
W44	W39	4
W44	W53	4
mean±SE		4.3±0.2

Co-cultivation of normal lymphocytes with thyrocytes from normal allogeneic foetal lambs resulted in severe damage to the thyrocyte monolayer in all 7 cases.

Table 25. The effect of co-cultivating autologous thyrocytes with lymphocytes from foetal lambs that had been submitted to thyroidectomy and allogeneic thyroid implantation.

Ewe bearing foetal lamb	Grade of cytotoxicity
Y260	4
Y270 (left foetus)	5
Y274 (left foetus)	5
Y284	4
Y296	5
Y318	4
W18[1]	2
W86	5
W201	4
W218	5
Or992	4
mean±SE	4.5[1]±0.2

Note: 1. Mean grade of 4.5 was calculated from 10 out of 11 cases, excluding number W18.

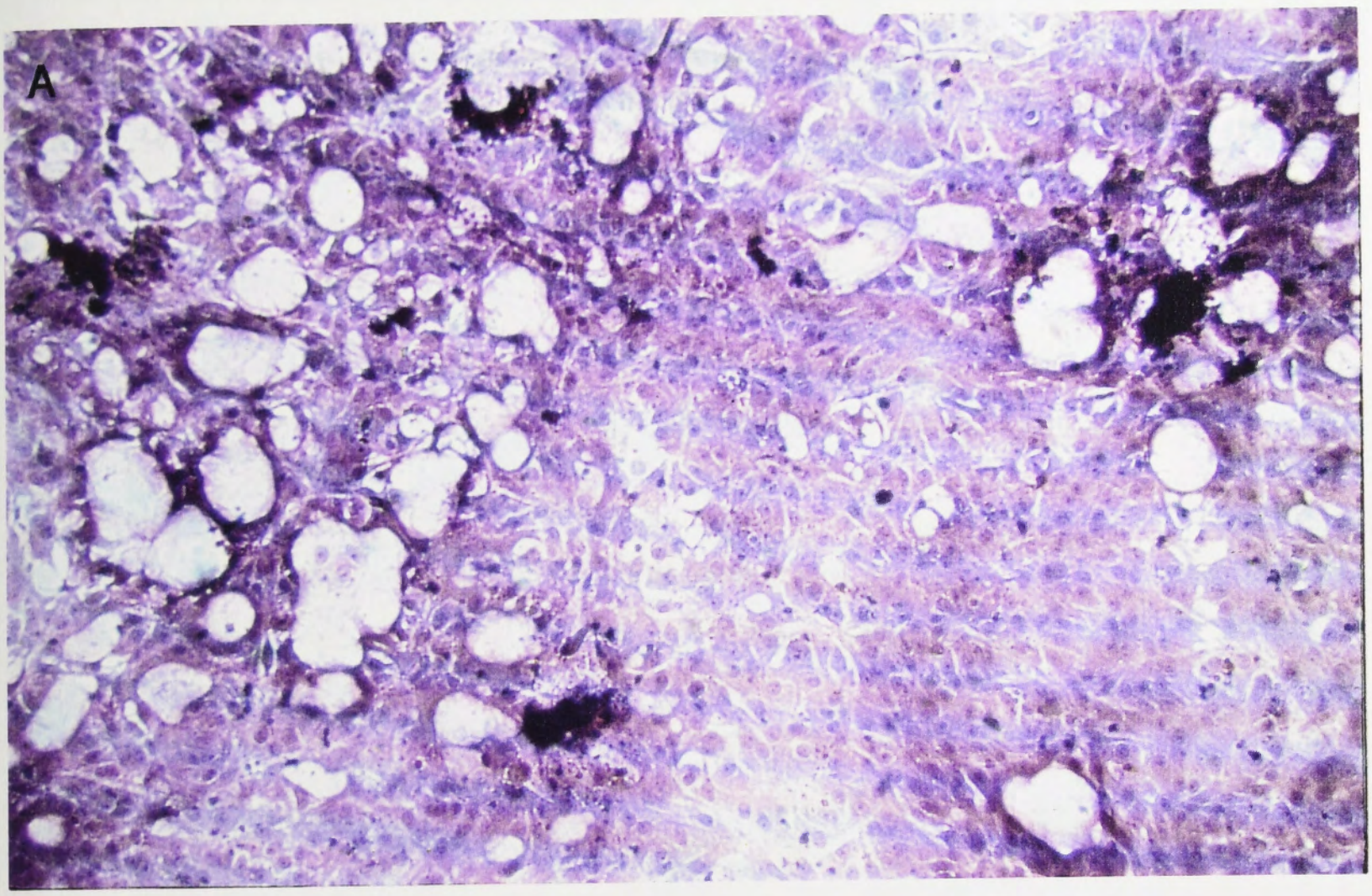
Eleven foetal lambs that had received thyroid allografts after thyroidectomy at 54 days were examined. Ten out of the 11 showed severe damage to the cultivated thyrocytes. The thyrocytes from foetus W18 retained a normal follicle structure and only slight follicle damage occurred. In this case, residual self thyroid tissue was found at *post mortem.*

Figure 31. Response of lymphocytes from thyroidectomized and thyroid-allografted foetal lambs to thyrocyte monolayers.

(In A and B, lymphocytes were collected from foetus W86 that had been thyroidectomized and had received thyroid allografts at 54 days gestation. Morphologic examination in this figure were performed 10 days after adding lymphocytes to thyrocyte monolayers. The coverslips bearing the indicated cells were all stained by means of H&E stain. Magnification ×195.)

(A) *Lymphocytes from foetal lamb W86, previously submitted to thyroidectomy and allogeneic thyroid implantation, were incubated with autologous thyrocytes.* Severe damage to the thyrocyte monolayer was observed. (Grade 5).

(B) *Lymphocytes from foetal lamb W86 previously submitted to thyroidectomy and allogeneic thyroid implantation were incubated with thyrocytes from the allograft donor.* The follicular structure remained intact in most parts of the thyrocyte monolayer. (Grade 1).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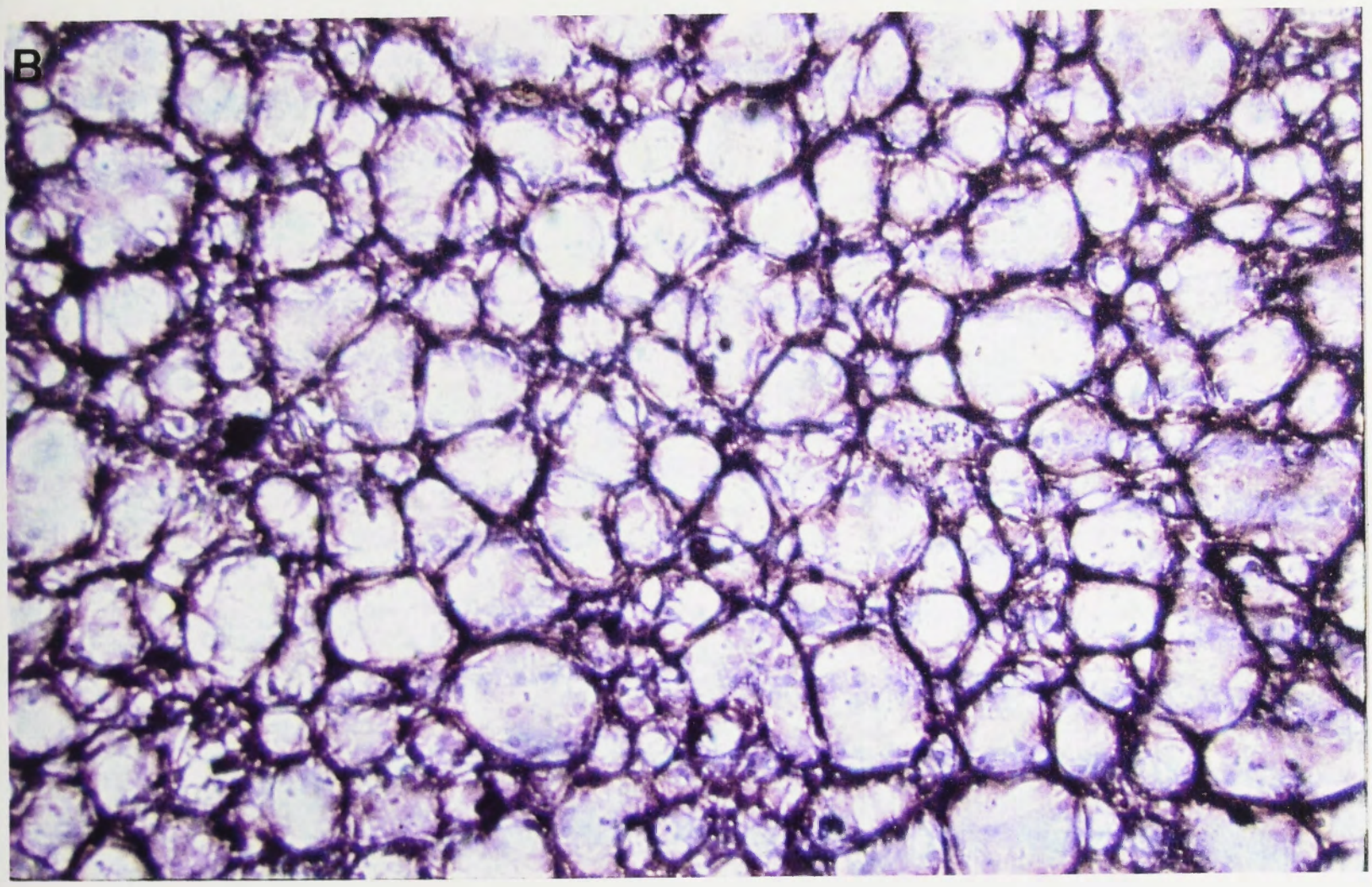
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Table 26. The effect of co-cultivating thyrocytes from the foetal donor of a thyroid lobe, transplanted to an allogeneic foetal recipient following thyroidectomy at 54 days gestation, with lymphocytes from the foetal lamb recipient of the thyroid allograft.

Ewe bearing recipient foetal lamb	Ewe bearing donor foetal lamb	Grade of cytotoxicity
Y274 (left foetus)	Y274 (right foetus)	1
Y284	Y287	2
Y296	W233	3
Y318	Y320	2
W18	W44	1
W86	W92	1
W201	W196	2
W218	W217	3
Or992	Or959	4
mean±SE		2.1±0.4

Nine thyroidectomized foetal recipients of thyroid allografts were examined. Six out of the 9 (66%) tested cases retained normal follicular structures with only slight damage to the thyroid monolayer. Two out of the 9 cases showed moderate damage. Only one case showed severe damage to the thyroid monolayers.

Table 27. The effect of co-cultivating thyrocytes from an allogeneic (third party) foetus with lymphocytes from a thyroidectomized and thyroid allografted foetus.

Ewe bearing thyroidectomized and thyroid allografted foetal lamb	Ewe bearing allogeneic donor foetal lamb	Grade of cytotoxicity
Y260	Y256	3
Y284	Y280	4
W86	W87	5
W86	W97	5
W201	Y280	4
Or992	Y317	4
mean±SE		4.2±0.3

Six thyroid donor / reecipient combinations of foetal lambs were tested. The cultivated thyroid monolayer in all of the tested cases exhibited severe damage after incubation with lymphocytes from thyroidectomized-allothyroid-implanted foetal lambs.

Table 28. Comparison of the expression of MHC class II antigen on the surface of thyrocytes after incubation with lymphocytes from ^{131}I exposed and normal DA rats.

Normal DA rat	Frequency of class II positive thyrocytes (%)	^{131}I exposed DA rat	Frequency of class II positive thyrocytes (%)	Frequency of class II expression by thyrocytes cultivated alone (%)
5	0	1/5	25	0
6	4	2/5	17	0
8	0	1/7*	0	0
9	1	2/7	20	0
10	0	3/7	20	0
11	3	4/7	23	0
12	0	1/4	14	0
13	0	2/4	10	0
14	0	1/9*	0	0
15	0	2/9*	0	0
mean±SE	0.8±0.5	mean±SE	12.9±3.1	0

Lymphocytes from 10 ^{131}I exposed and 10 normal DA rats have been examined for their capacity to induce class II antigen expression by co-cultivated syngeneic thyrocytes. Cultivated thyrocyte monolayers alone were also observed. After incubation for 48 hours with lymphocytes from ^{131}I exposed rats, a mean of 12.9%±3.1% thyrocytes showed positive staining for class II antigen. In contrast, after incubation with normal rat lymphocytes, only 0.8%±0.5% thyrocytes displayed positive staining. Cultivated thyrocytes alone displayed completely negative staining in all cases tested. (*) In the case of ^{131}I exposed rat 1/7, severe damage of the cultivated thyrocytes was not observed after incubation with its lymphocytes. In the case of rats 1/9 and 2/9, staining was only done at the third and fifth days of the incubation. As indicated in the notes to Table 29, class II antigen expression was not detected after periods of cultivation in excess of 48 hours.

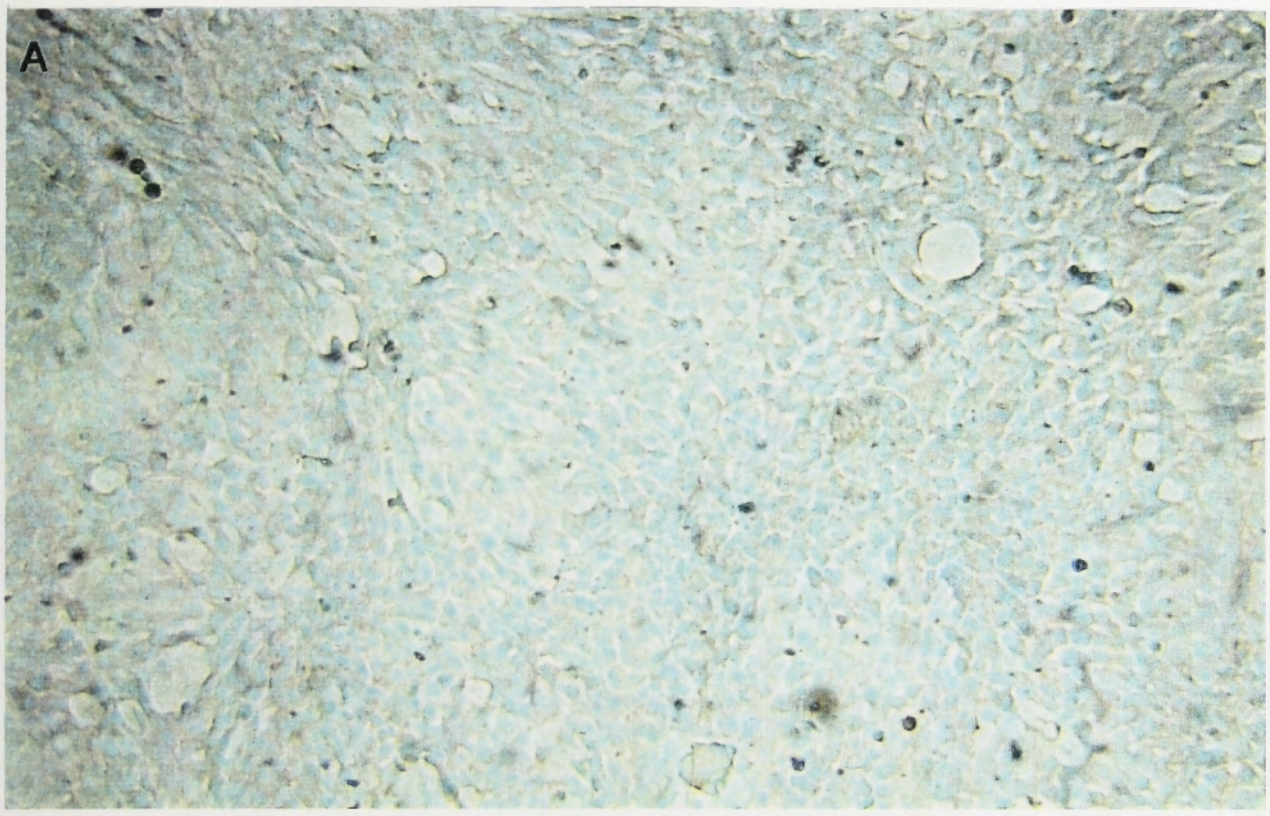
Figure 32. The expression of MHC class II antigen on DA rat thyrocytes.

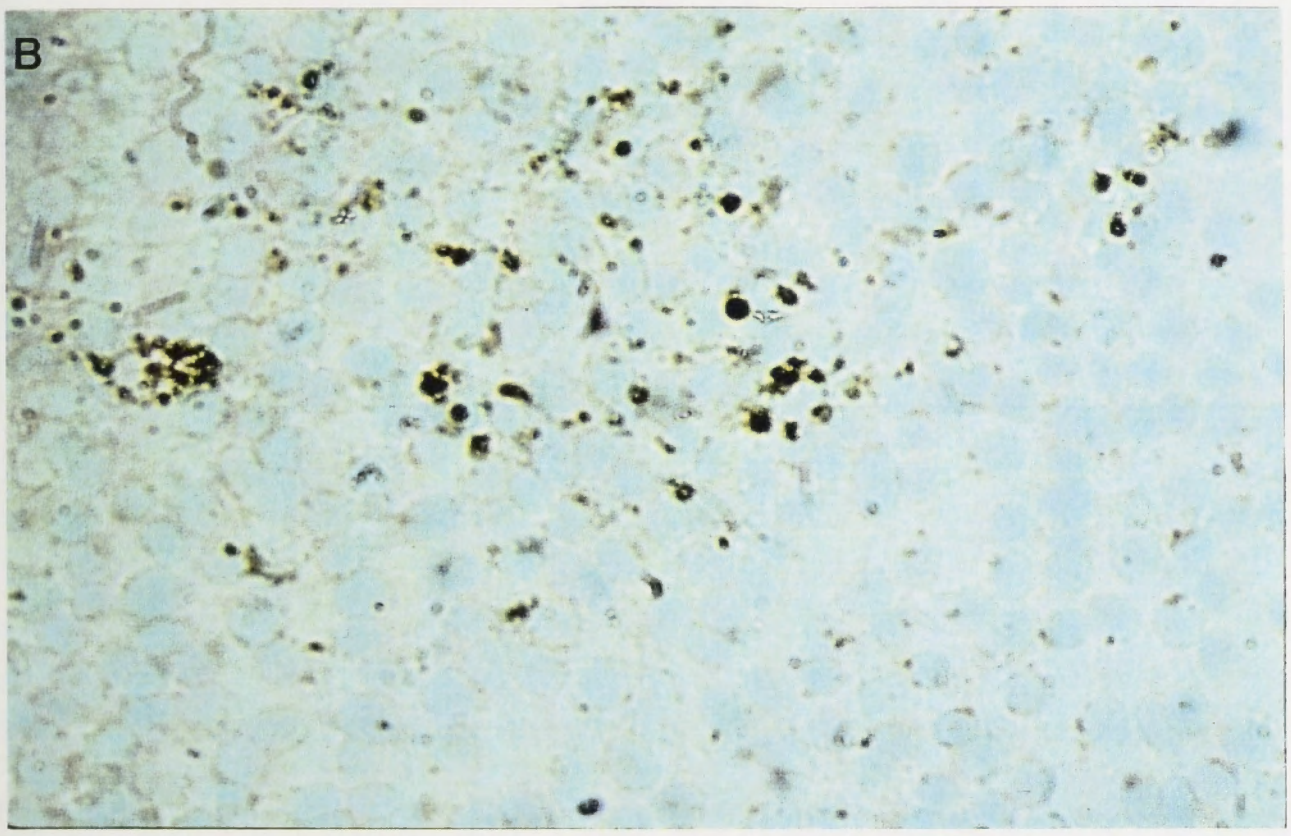
(The results in this figure were obtained from an ^{131}I exposed and a normal DA rat. The expression of class II antigen on the thyrocyte monolayer was examined by means of the avidin-biotin immunocytochemical method 24 hours after adding lymphocytes to thyrocyte monolayers. Counter staining was performed with methyl-green pyronin. Magnification ×292, unless otherwise stated).

(A) *Thyrocytes cultivated alone.* Staining of the cultivated thyrocyte monolayer for MHC class II antigen gave a negative result. (Magnification ×117).

(B) *Thyrocytes incubated with syngeneic lymphocytes from normal DA rats.* Staining of the cultivated thyrocyte monolayer for MHC class II antigen was negative. A positive result for MHC class II antigen was observed in some lymphocytes on the thyrocyte monolayer.

(C) *Thyrocytes incubated with syngeneic lymphocytes from ^{131}I exposed DA rats.* Staining of the cultivated thyrocyte monolayer for MHC class II antigen gave a positive result.


A


B

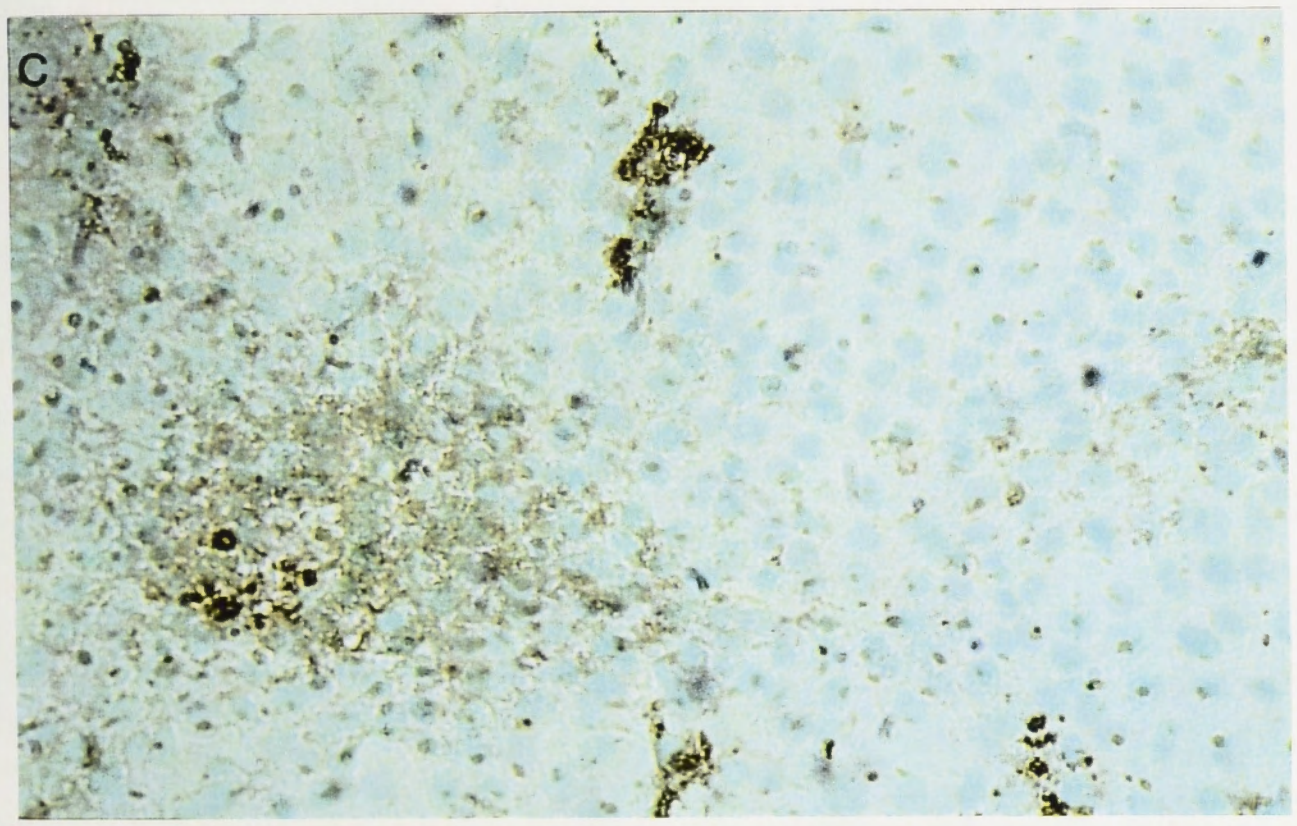

C

Table 29. The influence of duration of cultivation of thyrocytes with lymphocytes from ^{131}I exposed rats on their expression of class II antigen.

^{131}I exposed rat	Percentage of thyrocytes expressing class II antigens after various intervals (hours)		
	24	48	120
1/5	25	15	0
2/5	17	10	0
1/7	0	NT[1]	0
2/7	20	NT	0
3/7	20	NT	0
4/7	23	NT	0
1/4	NT	13	0
2/4	NT	10	0

Note 1. not tested.

Class II antigen expression on thyrocytes was influenced by the preceding duration of culture with autoreactive lymphocytes. Class II antigen was clearly expressed within 24-48 hours, but not after longer intervals.

Figure 33. Absence of a specific protein band from the supernatant when syngeneic or autologous thyrocytes were cultivated with lymphocytes from ^{131}I exposed rats or thyroidectomized foetal lambs, respectively.

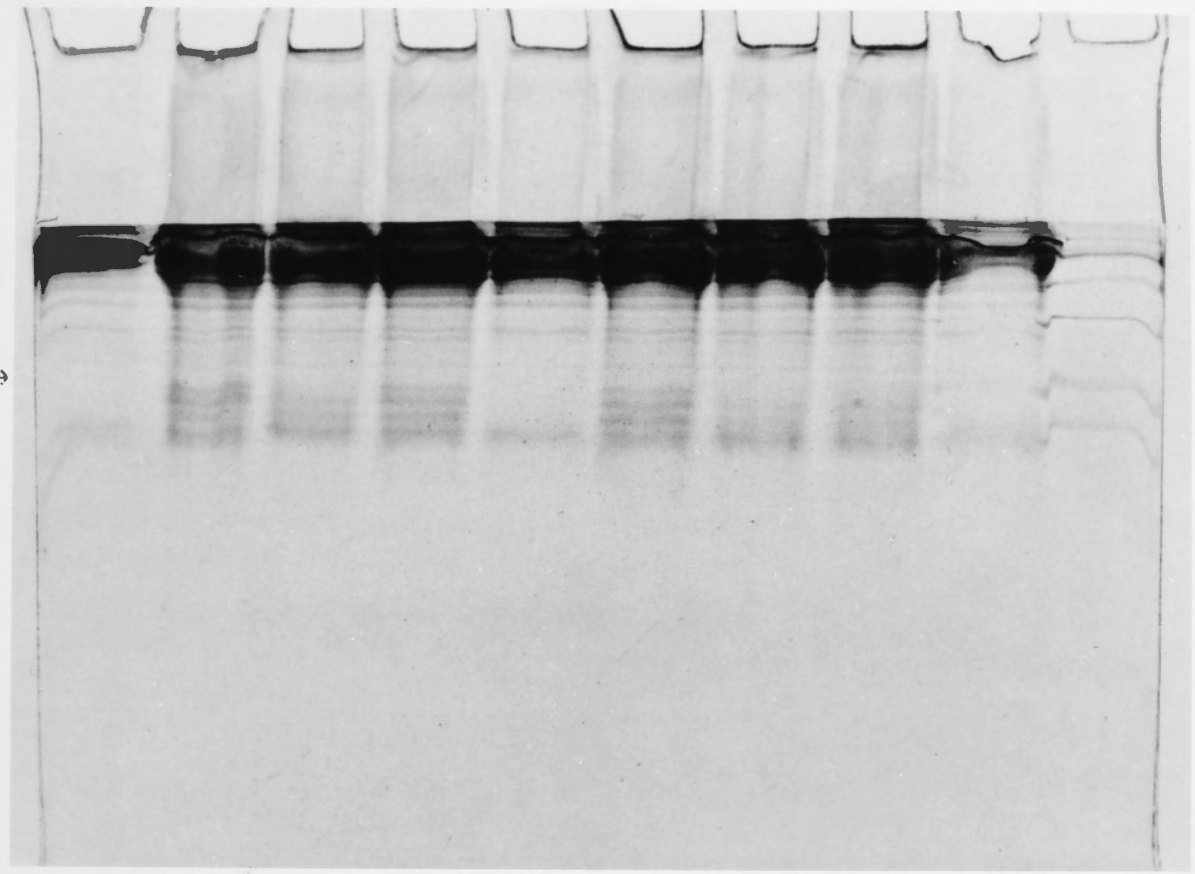
Analysis of molecules released into the supernatant when lymphocytes were cultured with thyrocytes was performed by means of SDS-PAGE as described by Schagger *et al.* (1987). The separating gels shown in (A) and (B) in this figure were prepared at a concentration of 10% total monomer (%T) and 3% crosslinking monomer (%C), while the stacking gels were made at a concentration of 4%T and 3%C.

Key: **1** Molecular weight standards (ranges: 14,400-97,400).
2 Culture medium alone.
3. Supernatant of thyrocytes cultivated alone.
4 Supernatant of thyrocytes cultivated with 10^7 normal lymphocytes.
5 Supernatant of thyrocytes cultivated with 10^7 lymphocytes from ^{131}I exposed rats or thyroidectomized foetal lambs.
6 Supernatant of thyrocytes cultivated with 7×10^6 lymphocytes from normal rat or foetal lamb and 5×10^6 lymphocytes from ^{131}I exposed rats or thyroidectomized foetal lambs.

(A) *Supernatant of cultivated cells from DA rats.* A protein band of approximately 17.6kD was lacking from the supernatant of thyrocytes cultivated with lymphocytes from ^{131}I exposed rats (No.5). It could be clearly observed in the supernatant from thyrocytes cultivated with normal lymphoytes (No.4) or from thyrocytes cultivated with a mixture of lymphocytes from normal and ^{131}I treated rats (No.6). This protein band was completely absent in the supernatant from thyrocytes cultivated alone (No.3) and in culture medium alone (No.2).

(B) *Supernatant of cultivated cells from foetal lambs.* A protein band of approximately 17.6kD was lacking from the supernatant of autologous thyrocytes cultivated with lymphocytes from thyroidectomized foetal lamb (No.5). It could be clearly observed in the supernatant of thyrocytes cultivated with lymphocytes from normal foetal lambs (No.4) or with a mixture of lymphocytes from normal and thyroidectomized foetal lambs (No.6). This protein band was completely absent from the supernatant of thyrocytes alone (No.3) and in culture medium alone (No.2).

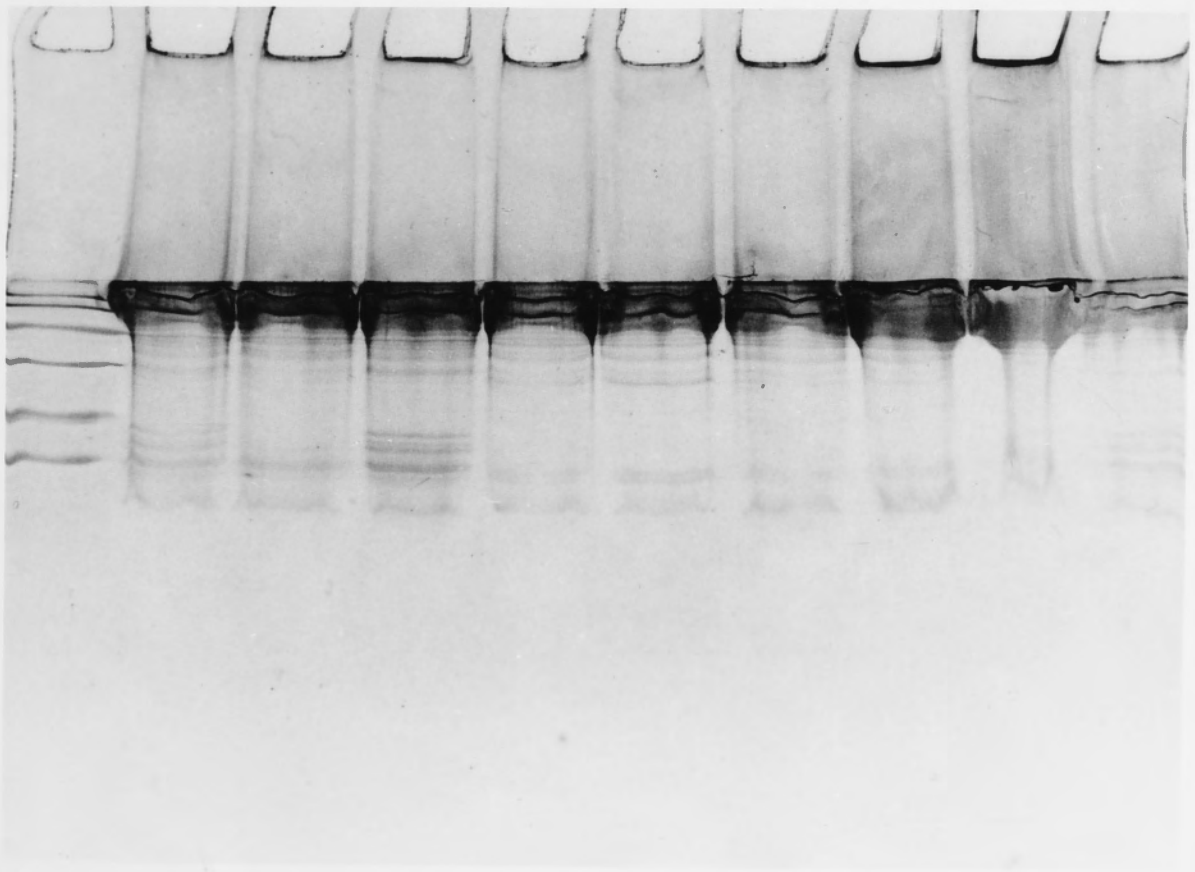
(A) 3 4 5 6 3 4 5 6 2 1



17.6kD→

←21kD

(B) 1 4 5 6 2 3 5 5 5 4



21kD→
17.6kD→

Figure 34. A comparison of occurrence of a 17.6kD protein band in supernatants of lymphocytes from different lymph organs of normal DA rats.

(The method of SDS-PAGE used in this figure was similar to that described in figure 33. Lymphocytes from the indicated tissues were cultivated alone for a period of 5 days before the supernatant was harverted)

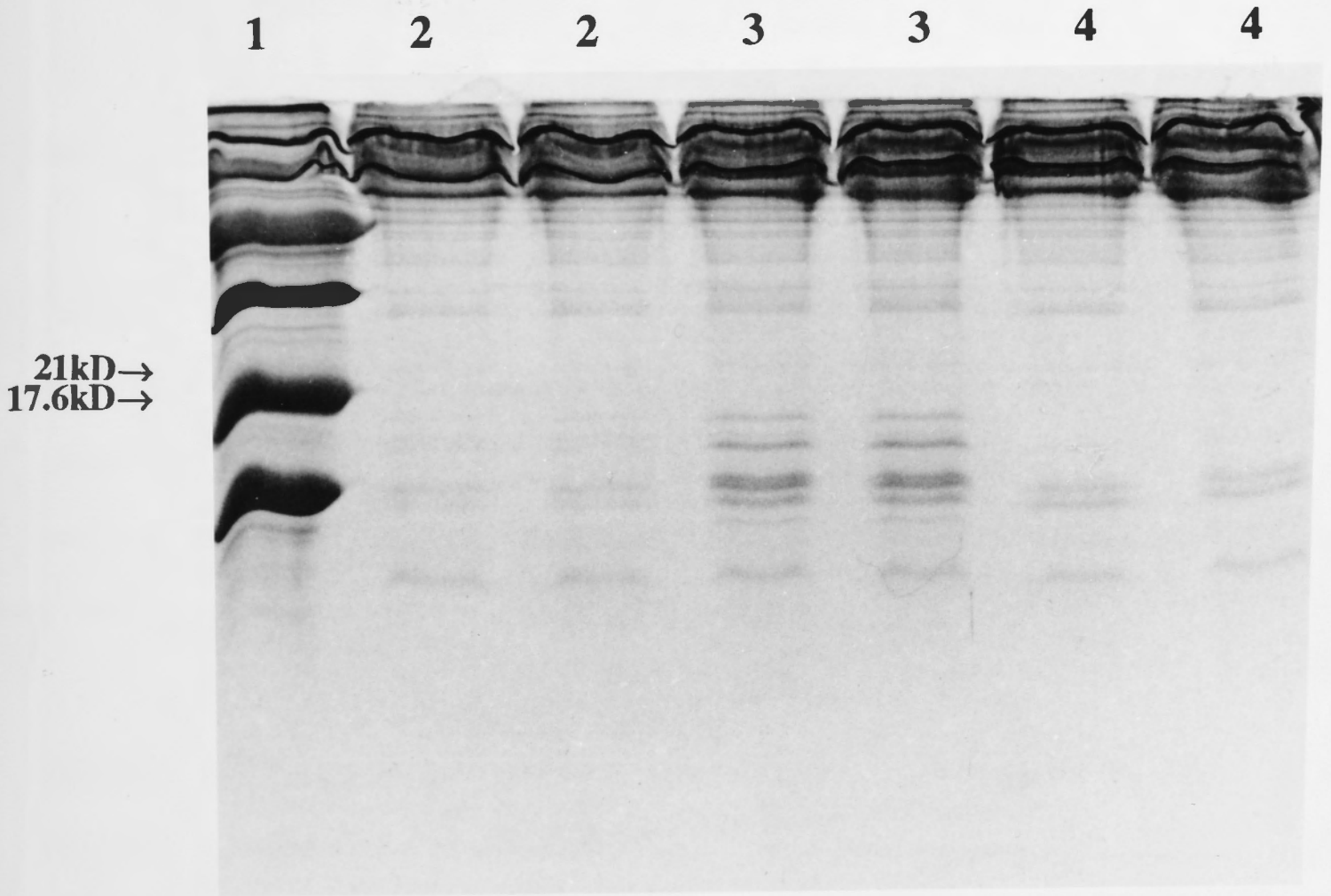
Key : **1** Molecular weight standards (range: 14,4000-97,400).

2. Supernatant of cultivated lymphocytes from lymph node.

3. Supernatant of cultivated lymphocytes from thymus

4 Supernatant of cultivated lymphocytes from spleen.

The protein band around 17.6kD was detected in the supernatant of lymphocytes from thymus (No.3) and lymph nodes (No.2), but was lacking from the supernatant of lymphocytes from the spleen (No.4).


1
2
2
3
3
4
4
21kD→
17.6kD→

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Appendix

The reproducibility of the gradings given to thyrocyte monolayers carrying lymphocytes from differing sources was tested by re-scoring the preparations from some experiments after concealing their identifying features. The outcome of "blind" scoring on some of the thyrocyte monolayers on which the results in Table 15 and 22 in the text were based is presented both as "raw data" and as re-assembled Tables.

Blind Test for Grade of Damage to Thyrocyte Monolayer for DA Rats

Blind code	Blind grade	Corresponding sample
2	4	NTM+1/11ALNC
3	1	NTM only (for 1/5)
4	1	NTM only (for 4/1)
6	1	NTM only (for 2/7)
7	1	NTM+NLNC (for 4/1)
10	2	NTM+NLNC+2/7ALNC
11	1	NTM+NLNC (for 1/5)
12	1	NTM+NLNC+2/11ALNC
15	1	NTM only (for 1/11,2/11)
16	5	NTM+4/1ALNC
19	5	NTM+1/13ALNC
20	1	NTM+NLNC+1/4ALNC
22	1	NTM+NLNC (for 1/11,1/12)
26	1	NTM+NLNC (for 1/13,2/13)
27	5	NTM+2/5ALNC
28	1	NTM+NLNC (for 2/7)
29	5	NTM+1/5ALNC
30	1	NTM+NLNC+1/5ALNC
32	1	NTM+NLNC (for 2/5)
33	2	NTM+NLNC+2/5ALNC
34	5	NTM+1/4ALNC
35	2	NTM+NLNC+1/11ALNC
37	1	NTM+NLNC+1/13ALNC
38	1	NTM only (for 2/5)
39	1	NTM+NLNC(for 1/4)
40	2	NTM+NLNC+4/1ALNC
41	5	NTM+2/11ALNC
42	5	NTM+2/7ALNC

Note: 1. TM — Thyroid monolayer.
2. NLNC — Normal lymph node cell.
3. ALNC — Autoimmune lymph node cell.
4. Date on which Table 15 was based.

Blind Test for Grade of Damage to Thyrocyte Monolayer for Foetal Lambs

Blind code	Blind grade	Corresponding sample
1	5	P118TM+NLNC($CD8^-$)+ALNC
5	4	P8TM+ALNC
8	5	P8TM+NLNC($CD4^+$)+ALNC
9	5	Or849TM+ALNC
13	2	P118TM+NLNC+ALNC
14	5	P8TM+NLNC($CD8^-$)+ALNC
17	5	Or849TM+NLNC($CD4^-$)+ALNC
18	1	P8TM+NLNC+ALNC
21	1	P118TM+NLNC($CD8^+$)+ALNC
23	1	Or849TM+NLNC($CD8^+$)+ALNC
24	5	P8TM+NLNC($CD4^-$)+ALNC
25	4	P118TM+ALNC
31	1	Or849TM+NLNC+ALNC
36	2	P8TM+NLNC($CD8^+$)+ALNC

Note: 1. TM — Thyroid monolayer.
2. NLNC — Normal lymph node cell.
3. ALNC — Autoimmune lymph node cell.
4. Date on which Table 22 was based.

Table 15. The effect of lymphocytes from normal and ^{131}I exposed DA rats on syngeneic thyrocyte monolayers.

^{131}I exposed rat	Grade of damage to thyrocyte monolayer		
	Normal rat lymphocytes	^{131}I exposed rat lymphocytes	Mixture of lymphocytes from ^{131}I exposed and normal rat
4/1	1	5	2
1/5	1	5	1
2/5	1	5	2
2/7	1	5	2
1/4	1	5	1
1/11	1	4	2
2/11	1	5	1
1/13	1	5	1
mean±SE	1±0	4.9±0.1	1.5±0.2

The effect of lymphocytes from 8 ^{131}I exposed and 8 normal DA rats was tested on syngeneic thyrocyte monolayers. Incubation with lymphocytes from ^{131}I exposed rats that had previously received implants of syngeneic thyroid resulted in severe damage to thyrocytes (mean grade 4.9±0.1). However, thyrocytes retained their normal structure after incubation with lymphocytes from normal syngeneic rats (mean grade 1±0). After incubation with mixtures of lymphocytes from both ^{131}I exposed and normal rats, damage to thyrocyte monolayers was curtailed (mean grade 1.5±0.2). The mean grade (1.5±0.2) induced by a mixture of lymphocytes was significantly lower ($P<0.01$) than the mean grade of 4.9±0.1 induced by lymphocytes from ^{131}I exposed rats.

Table 22. Influence of different T lymphocyte subsets from normal foetal co-twins on the cytotoxicity for autologous thyrocytes of lymphocytes from thyroidectomized foetal co-twins.

Ewe bearing identical twins	Grade of cytotoxic damage to thyrocytes: Lymphocyte subpopulation					
	Whole population cells from thyroidect-omized co-twin (5×10^6)	Mixture of whole population cells from normal and thyroidect-omized co-twin ($7{:}5\times10^6$)	Mixture of CD4 selected cells from normal and whole population cells from thyroidect-omized co-twin ($3{:}5\times10^6$)	Mixture of CD4 depleted cells from normal and whole population cells from thyroidect-omized co-twin ($7{:}5\times10^6$)	Mixture of CD8 selected cells from normal and whole population cells from thyroidect-omized co-twin ($2{:}5\times10^6$)	Mixture of CD8 depleted cells from normal and whole population cells from thyroidect-omized co-twin ($8{:}5\times10^6$)
Or849	5	1	NT[1]	5	1	NT
P8	4	1	5	5	2	5
P118	4	2	NT	NT	1	5
mean± SE	4.3±0.2	1.3±0.2	5±0	5±0	1.3±0.2	5±0

Note: **1.** Not tested.

After lymphocyte sorting, four lymphocyte subpopulations were obtained. Only that subpopulation of lymphocytes from the normal co-twin selected for expression of CD8 effectively curtailed autoreactivity on the part of cells from the thyroidectomized co-twin.